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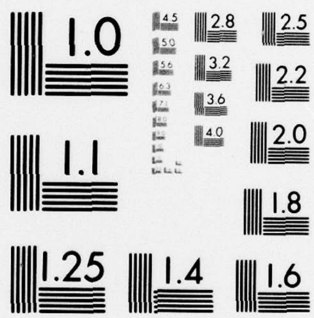

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Development of Short Term Immunotoxicological Assays for the Prediction of Chronic Toxicological Response Induced by Environmental Chemicals

Annual Report
September 1979
by

Albert E. Munson, Ph.D.

supported by
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-78-C-8083

Project Officer: Mary C. Henry, Ph.D.
Environmental Protection Research Division
U.S. Army Bioengineering Research and Development Laboratory
Fort Detrick, Frederick, Maryland 21701

Medical College of Virginia
Richmond, Virginia 23298

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The overall goal of the research is to systematically develop a battery of in vitro physical, biochemical, and functional assays to predict subchronic or chronic toxicologic behavior that would be produced by in vivo exposure to a chemical. The general experimental plan utilizes both in vivo and in vitro approaches. Results of in vivo tests are used as a reference to see if the in vitro tests can predict toxicologic responses. In vivo, mice were exposed subchronically to the environmental chemical trichloroethylene (TCE-2), and specific studies performed to assess bone marrow status, macrophage function, cell mediated immunity, hu-

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20. Abstract (continued)

moral immunity, and standard toxicologic parameters. In vitro, a tier assay system is being developed, including two cytotoxicity tests, assessment of DNA synthesis, phagocytosis, lymphocyte responsiveness to mitogens, enumeration of bone marrow stem cells, and an antibody-forming cell assay (the Mischell-Dutton assay).

The LD_{50}'s for male and female mice exposed by the gastrointenstinal tract to TCE-2 were 2402 and 2454 mg/kg, respectively. A combination range-finding study of TCE-2 in the drinking water and 30-day interaction with emulphor was performed. Considering only the lowest concentration of emulphor (1%), there were only four parameters significantly different from the appropriate control, but none of these parameters showed dose dependency and could not be specifically attributed to an interaction between TCE-2 and emulphor.

A subchronic four month study on trichloroethylene administered in the drinking water in concentrations between 0.1 and 5 mg/ml was performed in male and female CD-1 mice. In male mice, there was a trend towards a concentration-dependent increase in DNA synthesis. This trend was much more pronounced in female mice. Bone marrow stem cell number in female mice was decreased in a concentration-dependent fashion. There was a slight but significant decrease in bone marrow stem cells in male mice. Male mice showed no inhibition of delayed type hypersensitivity response, while females showed a 30-51% inhibition. This inhibition was not dose dependent and suggests that one particular cell type might be responsible for the effect. Antibody production as measured by spleen antibody-forming cells was not significantly altered in male mice, but showed a concentration-dependent suppression in females. No effect on recruitability was seen in male mice. In the males, all TCE-2-exposed groups showed a significant depression. The number of adherent cells from the peritoneal exudate cells provides an indicator of the number of macrophages in the peritoneal cavity. Female mice showed no change in the number of adherent cells, but the males showed a significant depression in the number of these cells. Chemotaxis ability of peritoneal exudate cells from the male mice exposed to TCE-2 showed a significant elevation over the vehicle controls. Phagocytosis of opsonized sheep erythrocytes by the adherent population of peritoneal exudate cells was suppressed in female mice, while that of the male mice was not altered.

The in vitro studies are conveniently divided into two parts--development studies on specific tier assays and the use of the assays in a combined exposure protocol. The actual decrease in cell number over time has proven to be a fairly reliable indication of cytotoxicity. Neutral red dye uptake into cells is presently being developed as a cytotoxicity assay. Measurement of the effects of chemicals on DNA synthesis is well developed and is represented by a Tier One assay which assesses the ability of a chemical to interfere with the incorporation of iododeoxyuridine (IUdR) into DNA.

The bone marrow stem cell assay is a Tier Three assay, and has developed more rapidly than the other assays during this contract period. We have determined that there is an important density dependency in terms of the number of cells plated in soft agar. The other in vitro assay that is well established is the phagocytosis of opsonized sheep erythrocytes by macrophages obtained from the peritoneal cavity. Thus far we have seen very little effect on either adherence or phagocytosis by in vitro exposure of macrophages to TCE-2 or dexamethasone.

Lymphocyte responsiveness to mitogens is another important immunologic assay which has been developed. We have selected Conconavalin A and bacterial lipopolysacchraide as the T lymphocyte and B lymphocyte mitogens, respectively. The results of bone marrow studies indicate that TCE-2 produces a dose- and time-dependent inhibition of DNA synthesis, bone marrow stem cell growth, and a decrease in cell number over time. Thus far in vitro and in vivo experiments show a correlation in the effects of TCE-2 on bone marrow cells. Concentrations as low as 10^{-5}M TCE-2 inhibit bone marrow stem cell growth in vitro, and there was a similar inhibition of stem cell growth in animals exposed in vivo to TCE-2 over a 4-month period.

EXECUTIVE SUMMARY

The overall goal of the research is to systematically develop a battery of *in vitro* physical, biochemical, and functional assays to predict subchronic or chronic toxicologic behavior that would be produced by *in vivo* exposure to a chemical. Use of such an *in vitro* set of tests will hopefully be quicker and less expensive than current methods using animals, and will provide valuable data which would facilitate the selection of chemicals for human use.

The general experimental plan utilizes both *in vivo* and *in vitro* approaches. Results of *in vivo* tests are used as a reference to see if the *in vitro* tests can predict toxicologic responses. *In vivo*, mice were exposed subchronically to the environmental chemical trichloroethylene (TCE-2), and specific studies performed to assess bone marrow status, macrophage function, cell mediated immunity, and humoral immunity. Standard toxicologic parameters such as body weight, organ weight, and hematologic status were also assessed to provide a reference for comparison to immune responses. *In vitro*, a tier assay system is being developed, which includes two cytotoxicity tests, assessment of DNA synthesis, phagocytosis, lymphocyte responsiveness to mitogens, enumeration of bone marrow stem cells, and an antibody-forming cell assay (the Mischell-Dutton assay).

The *in vitro* studies are conveniently divided into two parts--development studies on specific tier assays and the use of the assays in a combined exposure protocol. Various assays were utilized to evaluate the cytotoxic potential of chemicals on bone marrow, spleen, and thymus cells exposed *in vitro*. Extensive studies on trypan blue exclusion, ^{51}Cr release, and ^{111}Indium release proved these methods insufficiently sensitive for toxicologic assessment of a chemical. The actual decrease in cell number over time has proven to be a fairly reliable indication of cytotoxicity. Neutral red dye uptake into cells is presently being developed as a cytotoxicity assay. It appears that the neutral red dye uptake procedure can be developed into a sensitive indicator of cytotoxicity. Measurement of the effects of chemicals on DNA synthesis is well developed and is represented by a Tier One assay which assesses the ability of a chemical to interfere with the incorporation of iododeoxyuridine (IUdR) into DNA. In this assay, cells are incubated with ^{125}I-IUdR in microtiter plates over a three hour period, and the fentamoles of IUdR incorporated into the acid-precipitable fraction of the cells are assessed. Studies using flurodeoxyuridine (FUdR)

to enhance the incorporation of IUdR into DNA were performed. We concluded that FUdR is very effective in enhancing IUdR incorporation in bone marrow cells but not spleen cells; therefore, FUdR is used when bone marrow cells are employed, but not in experiments using spleen cells. DNA synthesis in bone marrow and spleen cells appears to be constant over a three hour period, but decreases to very low levels after 24 hours in culture. Because of this, all future experiments which rely on DNA synthesis as an indicator will have a maximum of three hours' exposure to the chemical.

The bone marrow stem cell assay is a Tier Three assay, and has developed more rapidly than the other assays during this contract period. We have determined that there is an important density dependency in terms of the number of cells plated in soft agar. The optimal cell concentration plated is between 5×10^5 cells and 1×10^6 cells.

The other in vitro assay that is well established is the phagocytosis of opsonized sheep erythrocytes by macrophages obtained from the peritoneal cavity. This is an important immunologic assay which detects the immunotoxicity of a chemical by assessing its ability to interfere with adherence and internalization of sheep erythrocytes by macrophages. This assay also will provide an indicator of the effect of the chemical on the ability of the macrophage to adhere to a substratum. Thus far we have seen very little effect on either adherence or phagocytosis by in vitro exposure of macrophages to TCE-2 or dexamethasone.

Lymphocyte responsiveness to mitogens is another important immunologic assay which has been developed over the last 8 months. The final outcome of this assay is that we are able to use ^{125}I-IUdR as the indicator of DNA synthesis induced by mitogens. We have selected Conconavalin A and bacterial lipopolysaccharide as the T lymphocyte and B lymphocyte mitogens, respectively. Generally, three concentrations of the mitogen are used, and we have been able to prepare the mitogens in microtiter dishes and freeze them until needed for a large experiment. The major problem that we are having with the mitogenicity assay is that the serum which supports the assay is extremely important; yet we have not been able to obtain a large batch of uniform serum preparation. We must select the best available serum for this assay. Another problem is that in about one out of every five experiments, there is no incorporation of the isotope. We believe that excess thymidine is entering the experiment, inhibiting ^{125}I-IUdR

incorporation. We are presently trying to find its source.

Experiments were conducted assessing cytotoxicity, DNA synthesis, and bone marrow stem cell growth from a single exposure of bone marrow cells. Five experiments have been performed using this method. Cells were exposed in flasks and samples removed for each of the assays at selected time intervals over 48 hours. The results of these studies indicate that TCE-2 produced a dose- and time-dependent inhibition of DNA synthesis and bone marrow stem cell growth. TCE-2 also produced a dose-dependent cytotoxicity as measured by a decrease in cell number over time. In contrast, dexamethasone concentrations between 10^{-2} and 10^{-5} produced no dose and time effect in bone marrow cells. We are continuing to refine the exposure method in the combined approach and believe that two chemicals can be examined per month in this system. Thus far in vitro and in vivo experiments show a correlation in the effects of TCE-2 on bone marrow cells. Concentrations as low as 10^{-5}M TCE-2 inhibit bone marrow stem cell growth in vitro, and there was a similar inhibition of stem cell growth in animals exposed in vivo to TCE-2 over a 4 month period.

In vivo, an acute toxicity study was performed to give an approximation of the toxicity of TCE-2. The LD_{50}'s for male and female mice exposed by the gastrointestinal tract to TCE-2 were 2402 and 2454 mg/kg, respectively. All TCE-2-caused deaths occurred within 24 hours of compound administration. The intended exposure route for environmental chemicals was via drinking water. A combination range-finding study of TCE-2 in the drinking water and a 30-day interaction with emulphor, the polyethoxylated vegetable oil which was used to maintain the trichloroethylene in aqueous suspension was performed. This study used two concentrations of TCE-2 (1 and 5 mg/ml) and four concentrations of emulphor (1%, 2%, 4%, and 10%). Body weight and fluid consumption were monitored during the study, and 31 toxicologic parameters were measured at the end of the study. Generally, both emulphor and TCE-2 decreased body weight as a function of concentration. Considering only the lowest concentration of emulphor, there were only four parameters significantly different from the appropriate control, but none of these parameters showed dose dependency and could not be specifically attributed to an interaction between TCE-2 and emulphor.

A subchronic four month study on trichloroethylene administered in the drinking water in concentrations between 0.1 and 5 mg/ml was performed in male and female CD-1 mice. A multivariate analysis was performed comparing the body weight changes of the treatment groups at the end of each week for eight weeks, adjusting for the covariate initial weight. Only minor differences

between the weight gains of the various groups were detected. The major reference toxicologic responses, i.e., organ weight, hematology, urinalysis, etc., are presently being analyzed. However, studies on bone marrow DNA synthesis, stem cell growth, antibody production, and cell mediated immunity haven been completed. In the male mice, there was a trend towards a concentration-dependent increase in DNA synthesis. This trend was much more pronounced in female mice. Bone marrow stem cell number in female mice was decreased in a concentration-dependent fashion. There was a slight but significant decrease in bone marrow stem cells in male mice as compared to the vehicle control, but this was not concentration dependent.

Cell mediated immunity was assessed by measuring delayed type hypersensitivity response to sheep erythrocytes. Male mice showed no inhibition of delayed type hypersensitivity response, while females showed a 30-51% inhibition. This inhibition was not dose dependent and suggests that one particular cell type might be responsible for the effect. Antibody production as measured by spleen antibody-forming cells was not significantly altered in male mice, but showed a concentration-dependent suppression in females.

Assays on recruitability, adherence, chemotaxis, and phagocytosis were performed on these mice exposed to TCE-2 for 4 months. No effect on recruitability was seen in female mice. In the males, all four TCE-2-exposed groups showed a significant depression as compared to the vehicle controls; however, this was not found to be concentration dependent. The number of adherent cells from the peritoneal exudate cells provides an indicator of the number of macrophages in the peritoneal cavity. Female mice showed no change in the number of adherent cells, but the males showed a significant depression in the number of these cells. Again, there was no dose dependency seen. Chemotaxis ability of peritoneal exudate cells from the male mice exposed to TCE-2 showed a significant elevation over the vehicle controls. The peritoneal exudate cells from the female mice showed no alteration. Phagocytosis of opsonized sheep erythrocytes by the adherent population of peritoneal exudate cells was suppressed in female mice, while that of the male mice was not altered.

Several more parameters await analysis; however, it is fairly clear that animals exposed to TCE-2 do have altered functions of bone marrow cells, humoral immunity, and cell mediated immunity. The functions of cells recruited into the peritoneal cavity are also altered. Only further studies will be able to explain the complexity of these responses in interaction.

Generally, the *in vivo* experiments where immunosupprensis was observed did not show good dose (concentration) dependency. This may be a function of the interaction among the lymphoid cells involved in the response or that the chemical induces its own metabolism.

The *in vitro* studies are not always showing concentration dependency. Again, the lymphoid cells cooperatively may be responsible. Also, the assays are still under refinement, and as they are better standardized, they may produce more dose dependency.

The pitfalls we have encountered were in the analytical area, where the use of emulphor has created problems in the analysis of chemicals. We have had difficulties in establishing a good cytotoxicity assay, but we are hopeful that the neutral red dye assay will supplement cell number as an appropriate measure of cytotoxicity. In conclusion, we feel that we have accomplished many of our goals this first year. We anticipate a six month exposure to TCE-2 to be complete in another month, enabling us to fully describe the *in vivo* effects of TCE-2 on bone marrow and spleen cells.

FOREWORD

This project is being conducted in the Division of Toxicology of the Department of Pharmacology of the Medical College of Virginia. Dr. Albert E. Munson is the principal investigator and is responsible for the overall activities of the project. Dr. Richard Carchman is a co-investigator and is responsible for the *in vitro* phagocytosis and chemotaxis investigation. Dr. Anthony Segreti was a co-investigator who helped in establishing the data storage retrieval and performed statistical analysis. Dr. Sung Choi, Professor of Biostatistics, took over these statistical responsibilities in April, 1979. Mr. Phillip Pollack developed and has implemented the Biostatistical Information System, of which this project is the major user. Ms. Virginia Sanders is responsible for many of the *in vitro* studies, including lymphocyte blastogenesis, neutral red dye uptake, and the *in vitro* exposure system. Ms. Beverly Barrett and Ms. Nedra Linder are responsible for exposing the animals, maintaining the animal records, and performing parts of the *in vivo* immunotoxicology studies. Ms. Patricia Hallett performs the bone marrow stem cell assays, and Ms. Bernadine Kauffman performs the DNA synthesis and antibody-forming cell assays. Mr. Robert Walter is an analytical chemist responsible for analyzing the drinking water and measuring the blood and tissue levels of the chemical under test.

The investigators conducting the research described in this report adhered to the Guide for Laboratory Animal Facilities and Care as provided by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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INTRODUCTION

The toxicological community is facing a major problem in fulfilling its responsibility to assess the toxic potential of chemicals before they are registered with federal regulatory agencies. The tests now used to make such assessments are expensive, time-consuming, and not always reliable.

The high costs of toxicological testing are well documented. Before a chemical can be registered for human consumption, acute, subchronic, and chronic toxicity studies must be performed in several species of animals at an average cost of more than $500,000 per compound (Mudel et al., Science, 193:834, 1976). Several government agencies conduct toxicological testing at an estimated cost in excess of $100 million, and private industry spends much more. Thus, a conservative estimate indicates that approximately 400 compounds can be evaluated per year with the funds derived from both the federal and private sectors. Data derived from the President's Advisory Committee (Handling of Toxicologic Information, Government Printing Office, Washington, D.C. 1966) indicate that thousands of chemicals generated from federal as well as private sources are in need of toxicological evaluation. Thus, current resources preclude the evaluation of most chemicals, many of which might be of great benefit or detriment to society.

Even if there were time and money enough to evaluate all chemicals being used, there are still complicated issues surrounding the use of *in vivo* tests as the sole basis for development of toxicological profiles: variations in test animals, species and strain differences in response to chemicals, and finally, meaningful extrapolations of these results to men.

The overall aim of this program is therefore to develop a system of *in vitro* assays that can be used to predict chronic or subchronic toxicological behavior produced by *in vivo* exposure to the chemical, and thereby determine not only which chemicals should be selected for future *in vivo* toxicological studies but also what types of studies should be undertaken. This *in vitro* system will allow for the interfacing of biotransforming capability and possibly for the use of fluids from animals exposed to the chemical. We also recognize that a comprehensive *in vitro* system requires a strong biochemical basis. A chemical that interferes with high-level cell functions involving specialized, differentiated activities such as antibody synthesis, neurotransmitter production, or insulin synthesis and/or activity, may or may not affect basic functions common to all cells such as cell growth, cell survival, enzyme activities, or macromolecular synthesis. Therefore,

it is important to develop an approach that considers both basic and specialized cell functions. The tier system for in vitro testing that is being developed in our research program is based on this kind of approach. We are proposing three tiers to be developed according to a sequential level of cell function in the immune response. Thus, the tier approach should provide for increasing sensitivity to perturbation by a chemical, i.e. the higher the tier assays, the greater number of biochemical sites that are available for alteration.

At the present stage of development, the Tier One assays are aimed at measuring the "cytotoxic" effects of the chemicals, the effects on DNA synthesis of resting and stimulated cells, and one cellular function, i.e. phagocytosis. The Tier Two assays involve recognition and functional responses and employ the mixed lymphocyte reaction and macrophage chemotaxis. The Tier Three assays require the formation of a product, i.e. stem cell growth requires new cells to be formed and the in vitro antibody assays measure the formation of a specific antibody. (This does not suggest that the lower tier assays do not result in product formation, for indeed they do, i.e. formation of DNA. The products formed in Tier Three assays are end line products and require many more steps.)

The specific objectives for the first year of the project are as follows:

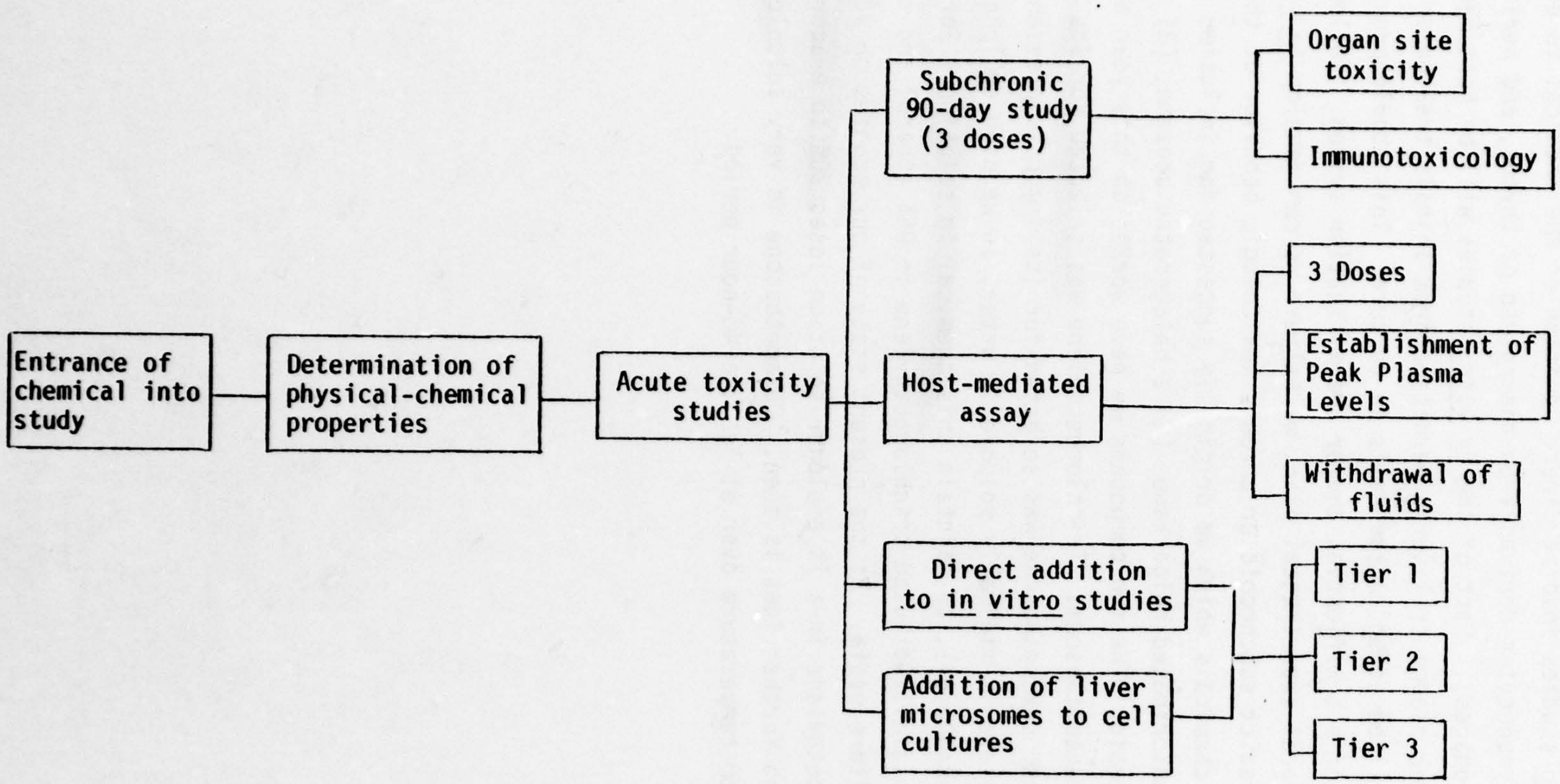
1. Design and develop an in vitro procedure for exposing volatile and non-volatile chemicals to bone marrow cells, lymphocytes, and macrophages.
2. Develop at least two assays that can be defined as cytotoxicity assays.
3. Develop two biochemical assays that would signal altered macromolecular metabolism.
4. Develop two functional assays that would predict an adverse effect in animal models.
5. Design, develop, and implement subchro..ic 90-day toxicological studies on chemicals administered in the drinking water that would provide reference in vivo data for the in vitro investigations.
6. Begin to develop analytical (i.e. gas chromatograph) capabilities for both the in vivo and in vitro aspects of the program.

The pathway of investigation for these studies is outlined in the chart on page 5. After the chemical has been selected on the basis of any number of criteria, including environmental importance, the known toxicological effects of the chemical, or mechanisms of its toxic properties, the physical-chemical nature of the chemical is characterized. Solubility properties and stability properties are then described for appropriate intervention into in vivo and in vitro studies. Next a straightforward acute toxicity study is done using the oral route of administration, and then,

based on this acute toxicity data, a subchronic study up to six months is performed to identify the organ site toxicity and characterize the immunotoxicologic parameters. The *in vitro* studies involve direct addition of the chemical to either bone marrow cells, lymphocytes derived from the spleen or thymus, and peritoneal exudate cells (macrophages). Part of the *in vitro* studies will be host-mediated assays in which either plasma or liver supernatant from animals treated or untreated with the chemical will be added to the cells in culture. This should provide the first approximation of a toxification and/or detoxification system. Following this pathway within a general experimental plan, we believe we can develop *in vitro* systems that will predict subchronic or chronic toxicologic behavior of the chemical.

The classes of chemicals which we originally suggested for inclusion in these studies were (1) a chlorinated haloalkane, (2) a halogenated benzene, (3) a heavy metal, and (4) a steroid. The two compounds we have worked on this year are trichloroethylene and dexamethasone. Trichloroethylene was selected for its environmental importance, and dexamethasone was selected for its immunosuppressant properties. Trichloroethylene is minimally soluble in water, so we used emulphor, a polyethoxylated vegetable oil, to maintain this chemical in solution. For the *in vitro* studies, we initially diluted trichloroethylene in 95% ethanol and then added it to the tissue culture media. At the present state of our studies on stability, 5 mg/ml of trichloroethylene in a 1% emulphor solution loses 30% to evaporation within 5 hours. However, no further loss is seen. Dexamethasone is very soluble in water, and is stable at room temperature over at least a 48-hour period.

PATHWAY OF INVESTIGATION



PART 1 - IN VIVO

TRICHLOROETHYLENE IN VIVO

Acute Toxicity of Trichloroethylene in CD-1 Male and Female Mice

Introduction

This study was undertaken as part of an overall toxicologic evaluation of trichloroethylene. Trichloroethylene was first synthesized by Fisher in 1864. Commercial production in the United States started in 1925, and consumption grew to 500-600 million pounds in the period from 1965 to 1970. Although trichloroethylene was once used as an anesthetic and now serves as an extraction-solvent in food processing, it is primarily used for metal degreasing operations in diverse industries. We may assume that eventually the environment acquires all the produced trichloroethylene, most of it in the form of waste solvents deposited by these industries in sewers and/or land fills. Thus, trichloroethylene is of current interest as a potential toxicological problem. The Environmental Protection Agency reports that drinking water contains as much as 5 μg/liter.

Animals and Housing

The animals used in the study were six-week old male and female CD-1 ICR mice obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Quarantine lasted 3 days. The mice were housed 6 per cage on sawdust bedding in animal rooms maintained at 70-74° F and 40-60% relative humidity with a 12-hour light-dark cycle. Purina mouse chow and tap water were available ad libitum, except during the 18-hour fasting period prior to administration of trichloroethylene.

Materials and Equipment

Trichloroethylene (certified ACS Lot 781557) was obtained from Fisher Scientific Company, Fairlawn, New Jersey, and prepared as a suspension in a 1:9 solution of emulphor and distilled water. Emulphor (EL620) was obtained from GAF Corporation, New York, New York. Concentrations of trichloroethylene were prepared so the desired dose could be delivered in a volume of 0.01 ml per gram of body weight. The suspensions were prepared on the day of administration and maintained in dark glassware at 4° C until used. Mice were gavaged by means of a 1-ml syringe and an 18-gauge needle.

Evaluation

After administration of the chemical, the mice were monitored for behavioral, pharmacologic, and toxicologic effects continuously for 4 hours, hourly for 8 hours, and then twice daily for 14 days. All mice dying were necropsied and gross pathology

described. LD_{50} and slopes with corresponding 95% confidence limits of the dose-response curves were calculated by method of Litchfield and Wilcoxan (J. Pharmacol. and Exp. Therap., 96:99-113, 1949) and LOG Probit Analysis (SAS PROC Probit: A.J. Barr et al., A User's Guide to SAS 76, 1976).

Results

Table 1 shows the acute toxicity data for trichloroethylene. No deaths occurred at doses up to 750 mg/kg for females and 1250 mg/kg for males; there was 100% mortality at doses of 5500 mg/kg for females and 6000 mg/kg for males. The LD_{50} with 95% confidence limits is 2454 mg/kg (2040-3062) for female mice and 2402 mg/kg (2065-2771) for male mice. The slope functions of the dose-response curves for female and male mice are 1.835 and 1.587 respectively.

The deaths occurred after 24 hours of trichloroethylene administration. Other responses observed were dependent on dose and time, and occurred in the following sequence: ataxia, loss of ability to stand, ruffled fur, loss of righting reflex, loss of response to tendon pressure, abdominal breathing, and death. No animals that recovered from anesthesia died during the 14-day observation period. The only gross pathology observed was hyperemia of the stomach of mice dying from lethal doses of trichloroethylene. Mice sacrificed at 14 days showed no gross pathology.

Discussion

Several reviews are available on the toxicology of trichloroethylene. The only acute oral toxicity reports on trichloroethylene were by Christensen, who showed that the LD_{50} in rats is 4920 mg/kg, and by the Carcinogenesis Bioassay Program, which indicates that the acute toxicity in mice is greater than 10,000 mg/kg. The discrepancy between our findings and the findings of the Carcinogenesis Bioassay Program report may be related to the vehicle used to administer trichloroethylene. As indicated above, we used emulphor, whereas the Carcinogenesis Bioassay Program used corn oil. Another difference between the studies is the strain of mouse. We used a Swiss albino random-bred mouse, while the Carcinogenesis Bioassay Program used a B6C3F1 mouse.

It appears that the site of action for the acute lethal response to trichloroethylene is central nervous system suppression. In contrast to the halomethanes (trichloromethane, dichlorobromomethane, dibromochloromethane, and tribromomethane) where mice died over a 9-day period, trichloroethylene causes death either within 24 hours or not at all within the 14-day observation period.

Table 1

Acute Toxicity (mg/kg) of Trichloroethylene in CD-1 Mice

Sex	LD_{10}	LD_{50}	LD_{90}	Slope	No. Mice
Females	1159 (795-1450)	2454 (2040-3062)	5197 (3939-8592)	1.835	121
Males	1356 (972-1645)	2402 (2065-2771)	5001 (4055-7250)	1.587	136

CD-1 male and female mice were fasted for 18 hours prior to a single gavage of trichloroethylene. The LD_{50}'s of the dose response curves were analyzed by LOG Probit Analysis (SAS LOG Probit) as described by Finney (Statistical Methods in Biological Assay, second edition. London: Griffin Press, 1971).

Trichloroethylene:Emulphor Interaction Study

Introduction

As described earlier, it was found necessary to combine trichloroethylene with emulphor in order to maintain it in drinking water. Before proceeding with a subchronic study of trichloroethylene, however, it was necessary to know if an interaction between the two would occur, if the emulphor itself would have adverse effects, and what the lowest concentration of emulphor would be that could be used.

Animals and Housing

Male and female CD-1 mice, 15 to 20 grams, were obtained from Charles River Laboratories and quarantined for 5 days. The mice were housed 5 per cage on sawdust in plastic cages with wire tops and were identified by ear markings. Purina Laboratory Chow and tap water were available *ad libitum*. These animals came in two boxes of 100 each and were randomly placed in their cages. An analysis of variance was performed on the initial weights of these mice. The analysis indicated that the mice had not been randomly distributed between groups. However, weights within a specific group were not statistically different from one another. Because of this dilemma, all mice are now routinely randomized by use of a special computer program and are analyzed for statistical differences prior to the start of a study.

Materials and Methods

After the quarantine period, groups of mice were exposed to emulphor concentrations of 10%, 4%, 2%, or 1%,and concentrations of trichloroethylene at 1 mg/kg and 5 mg/kg. Tap water was used as a diluent. Fluid consumption was measured three times a week by weighing the bottle, subtracting the difference between the weights, and dividing the grams consumed by the number of mice and the number of days. Body weights were determined three times weekly using a Mettler top-loading balance accurate to 0.1 grams. Two cages were used for each of the 13 groups. At the end of the exposure period, 31 different variables were measured. An interaction analysis was used to determine the emulphor effects, trichloroethylene effects, and the interaction effects. Dunnett's T Test was used to determine if the individual groups were different from the appropriate control. The protocol and the standard operating procedures for each of the individual variables are provided in the Appendix.

Results and Discussion

A composite tabulation of the mean body weights and fluid consumption for mice receiving the four different concentrations of emulphor over the 30-day period is

provided in Table 2. For tap water (WTNA and FWTNA), weanling mice gained an average of 15.5 grams over the 30-day period and consumed 7.3-11.4 grams of water. From these data, it does not appear that the mice increased their fluid intake on a per gram basis. The relationship between weight gain and emulphor concentration is graphically represented in Figure 1. Because the randomization was not compete, a covariant analysis with a multivariate analysis was used to determine the emulphor effect on weight gain. The data indicate that emulphor at concentrations of 10% caused a slower rate of weight gain. The data in Table 2 on fluid consumption suggest that this slower rate is a function of fluid consumption, since the mice drinking the higher concentration of emulphor consumed less fluid. Furthermore, from this small amount of data, it appears that the mice receiving 1:50 or 1:100 emulphor:water consumed about the same amount as mice receiving the tap water.

Summary data and graphs showing the relationship between weight gain, fluid consumption, TCE-2 consumed, and emulphor concentrations are provided in Tables 3 and 4 and Figures 2 through 7. When mice received 1 mg/ml of TCE-2 added to the four different concentrations of emulphor, they showed a different pattern in fluid consumption and weight gain from those receiving just tap water. Figure 2 shows that mice receiving 1 mg/kg of TCE-2 in 1 % emulphor gained normally for about one week and then leveled off. However, the pattern for body weight gain when 1 mg/kg of TCE-2 is added to the other concentrations of emulphor was similar to tap water alone. Although TCE-2 in emulphor concentrations of 2 and 4% may have had some effect on body weight gain, the initial body weights of the groups receiving these concentrations were less than the other groups due to inadequate randomization. When 5 mg/ml of TCE-2 was delivered in the four emulphor vehicles, weight changes were more pronounced. Body weight gain was markedly suppressed with 10% emulphor and the least suppressed with 1% emulphor (Table 4 and Figure 3). It is quite clear that the decreased body weight is a function of decreased fluid consumption (Table 4). Animals receiving 5 mg/kg of TCE-2 in 10% emulphor have a markedly reduced fluid consumption; however, fluid consumption increased as the concentration of emulphor decreased. Animals receiving 5 mg/kg of TC-2 in 10% emulphor had a 34% decrease in fluid consumption per day when compared to the animals receiving tap water over the 30-day period. On the basis of mg/kg of TCE-2 consumed, there was no difference among the four emulphor groups (Table 4). The average TCE-2 intake was 1004 mg/kg for 10% emulphor, 1067 mg/kg for 4% emulphor, 939 mg/kg for 2% emulphor, and 1181 mg/kg for 1% emulphor.

We have also plotted the data as a function of TCE-2 concentration, keeping the emulphor vehicle constant. A summary of the data is provided in Tables 5 through 8, and the body weight gain graphs are in Figures 4 through 7. The available data and its constraints suggest that all 4 vehicle concentrations deliver about the same

Table 2

Tabulation of Body Weight and Fluid Consumption in CD-1 Male Mice Exposed to Varying Concentrations of Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
1:10 Emulphor									
a)	19.0	18.0	22.3	22.2	24.6	28.7	30.8	21.6	32.4
b)	-	3.3	7.2	5.0	8.5	4.9	7.2	7.5	8.2
1:25 Emulphor									
a)	17.7	20.4	23.5	23.9	26.3	29.3	31.0	32.0	32.8
b)	-	5.8	8.9	6.6	10.4	6.0	8.2	9.2	10.0
1:50 Emulphor									
a)	18.9	20.8	24.3	25.2	28.0	30.2	32.1	32.4	34.4
b)	-	6.9	9.4	6.5	11.4	5.7	8.6	8.9	9.8
1:100 Emulphor									
a)	20.5	23.4	26.1	27.4	29.4	31.7	32.6	33.6	33.1
b)	-	7.4	10.2	8.1	12.5	6.6	12.4	10.2	9.2

a) body weight in grams

b) mililiters of fluid consumed/day

Table 3

Tabulation of Body Weight, Fluid Consumption, and Trichloroethylene Consumption in CD-1 Male Mice Exposed to 1 mg/ml Triclorethylene in Varying Amounts of Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
c)	-	-	-	-	-	-	-	-	-
1 mg/ml TCE2 in 1:10 Emulphor									
a)	20.9	18.6	21.1	22.1	25.2	26.5	29.1	30.9	32.8
b)	-	2.7	5.7	4.1	7.6	3.7	6.6	7.0	7.8
c)	-	150	267	187	302	140	226	227	239
1 mg/ml TCE2 in 1:25 Emulphor									
a)	18.3	19.9	22.9	23.5	25.9	29.3	29.6	31.5	31.9
b)	-	5.3	8.9	5.6	9.4	5.4	6.7	8.0	7.0
c)	-	267	391	239	364	183	228	254	225
1 mg/ml TCE2 in 1:50 Emulphor									
a)	18.6	20.8	23.9	25.1	27.5	30.3	31.0	32.1	32.6
b)	-	6.2	9.0	6.3	12.0	6.1	8.6	8.6	10.4
c)	-	300	379	251	439	201	277	269	319
1 mg/ml TCE2 in 1:100 Emulphor									
a)	20.2	22.2	25.6	24.9	25.5	26.0	26.2	26.1	27.4
b)	-	4.3	7.0	5.8	8.7	5.4	5.6	5.3	6.9
c)	-	192	275	236	344	220	237	233	249

a) body weight in grams

b) milliliters of fluid consumed/day

c) mg/kg of TCE2 consumed/day

Table 4

Tabulation of Body Weight, Fluid Consumption, and Trichloroethylene Consumption in CD-1 Male Mice Exposed to 5 mg/ml Tricloroethylene in Varying Amounts of Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
c)	-	-	-	-	-	-	-	-	-
5 mg/ml TCE2 in 1:10 Emulphor									
a)	18.7	14.3	14.2	17.2	19.2	21.2	21.6	22.9	24.5
b)	-	1.8	3.7	4.5	3.9	4.1	3.4	3.8	4.4
c)	-	642	1364	1366	1078	1006	822	852	931
5 mg/ml TCE2 in 1:25 Emulphor									
a)	19.7	16.6	18.4	19.6	23.1	26.6	26.3	27.2	28.4
b)	-	2.8	4.6	3.5	8.4	5.2	4.2	4.5	5.8
c)	-	854	1278	909	1827	978	818	840	1030
5 mg/ml TCE2 in 1:50 Emulphor									
a)	20.2	18.9	22.0	20.6	23.4	26.2	26.2	27.2	27.5
b)	-	3.6	6.0	2.7	5.6	4.5	4.0	3.8	5.2
c)	-	963	1396	668	1219	864	759	700	942
5 mg/ml TCE2 in 1:100 Emulphor									
a)	19.2	18.6	22.8	23.3	25.2	29.0	28.1	29.9	30.8
b)	-	4.2	8.4	4.7	8.0	6.3	4.2	5.0	6.8
c)	-	1144	1857	1012	1614	1093	762	851	1116

a) body weight in grams

b) milliliters of fluid consumed/day

c) mg/kg of TCE2 consumed/day

Table 5

Summary Tabulation of Body Weight, Fluid Consumption, and Trichloroethylene Consumption in CD-1 Male Mice Exposed to 1 and 5 mg/ml Trichloroethylene in 1:10 Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
c)	-	-	-	-	-	-	-	-	-
a)	19.0	18.0	22.3	22.2	24.6	28.7	30.8	31.6	32.4
b)	-	3.3	7.2	5.0	8.5	4.9	7.2	7.5	8.2
c)	-	-	-	-	-	-	-	-	-
1 mg/ml TCE2 in 1:10 Emulphor									
a)	20.9	18.6	21.1	22.0	25.2	26.5	29.1	30.9	32.8
b)	-	2.7	5.7	4.1	7.6	3.7	6.6	7.0	7.8
c)	-	150	267	187	30.2	140	226	227	239
5 mg/ml TCE2 in 1:10 Emulphor									
a)	18.7	14.3	14.2	17.2	19.2	21.2	21.6	22.9	24.5
b)	-	1.8	3.7	4.5	3.8	4.1	3.4	3.8	4.4
c)	-	642	1364	1366	1078	1006	822	852	931

a) body weight in grams

b) milliliters of fluid consumed/day

c) mg/kg of TCE2 consumed/day

Table 6

Summary Tabulation of Body Weight, Fluid Consumption, and Trichloroethylene Consumption in CD-1 Male Mice Exposed to 1 and 5 mg/ml Trichloroethylene in 1:25 Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
c)	-	-	-	-	-	-	-	-	-
1:25 Emulphor									
a)	17.7	20.4	23.5	23.9	26.3	29.3	31.0	32.0	32.8
b)	-	5.8	8.9	6.6	10.4	6.0	8.2	9.2	10.0
c)	-	-	-	-	-	-	-	-	-
1 mg/ml TCE2 in 1:25 Emulphor									
a)	18.3	19.9	22.9	23.5	25.9	29.3	29.6	31.5	31.9
b)	-	5.3	8.9	5.6	9.4	5.4	6.7	8.0	7.0
c)	-	267	391	238	364	183	228	254	225
5 mg/ml TCE2 in 1:50 Emulphor									
a)	19.7	16.6	18.4	19.6	23.1	26.6	26.3	27.2	28.4
b)	-	2.8	4.6	3.5	8.4	5.2	4.2	4.5	5.8
c)	-	854	1278	909	1827	978	818	840	1030

a) body weight in grams

b) milliliters of fluid consumed/day

c) mg/kg of TCE2 consumed/day

Table 7

Summary Tabulation of Body Weight, Fluid Consumption, and Trichloroethylene Consumption in CD-1 Male Mice Exposed to 1 and 5 mg/ml Trichloroethylene in 1:50 Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
c)	-	-	-	-	-	-	-	-	-
1:50 Emulphor									
a)	18.9	20.8	24.3	25.2	28.0	30.2	32.1	32.4	34.4
b)	-	6.9	9.4	6.5	11.4	5.7	8.6	8.8	9.8
c)	-	-	-	-	-	-	-	-	-
1 mg/ml TCE2 in 1:50 Emulphor									
a)	18.5	20.8	23.8	25.1	27.4	30.2	31.0	32.1	32.6
b)	-	6.2	9.0	6.3	12.0	6.0	8.6	8.6	10.4
c)		300	379	251	439	201	276	269	319
5 mg/ml TCE2 in 1:100 Emulphor									
a)	20.2	18.9	22.0	20.6	23.4	26.2	26.2	27.2	27.5
b)	-	3.6	6.0	2.7	5.6	4.5	4.0	3.8	5.2
c)	-	963	1396	668	1219	864	759	700	942

a) body weight in grams

b) milliliters of fluid consumed/day

c) mg/kg of TCE2 consumed/day

Table 8

Summary Tabulation of Body Weight, Fluid Consumption, and Trichloroethylene Consumption in Male CD-1 Mice Exposed to 1 and 5 mg/ml Trichloroethylene in 1:100 Emulphor over Thirty Days

	Days								
	0	4	8	11	15	18	22	25	29
Tap Water									
a)	20.4	23.4	26.9	26.9	29.6	32.0	34.0	34.7	35.9
b)	-	7.8	9.1	7.3	11.4	10.6	8.1	8.7	9.4
c)	-	-	-	-	-	-	-	-	-
1:100 Emulphor									
a)	20.5	23.4	26.1	27.4	29.4	31.7	32.6	33.6	33.1
b)	-	7.4	10.2	8.1	12.5	6.6	12.4	10.2	9.2
c)	-	-	-	-	-	-	-	-	-
1 mg/ml TCE2 in 1:100 Emulphor									
a)	20.2	22.2	25.6	24.9	25.5	25.9	26.2	26.1	27.4
b)	-	4.3	7.0	5.8	8.6	5.4	5.6	5.3	6.8
c)	-	192	275	236	344	220	237	233	249
5 mg/ml TCE2 in 1:100 Emulphor									
a)	19.2	18.6	22.8	23.3	25.2	29.0	28.1	29.9	30.8
b)	-	4.2	8.4	4.7	8.0	6.3	4.2	5.0	6.8
c)	-	1144	1856	1012	1614	1092	762	851	1116

a) body weight in grams

b) milliliters of fluid consumed/day

c) mg/kg of TCE2 consumed/day

Figure 1
Body Weight Gain in CD-1 Male Mice

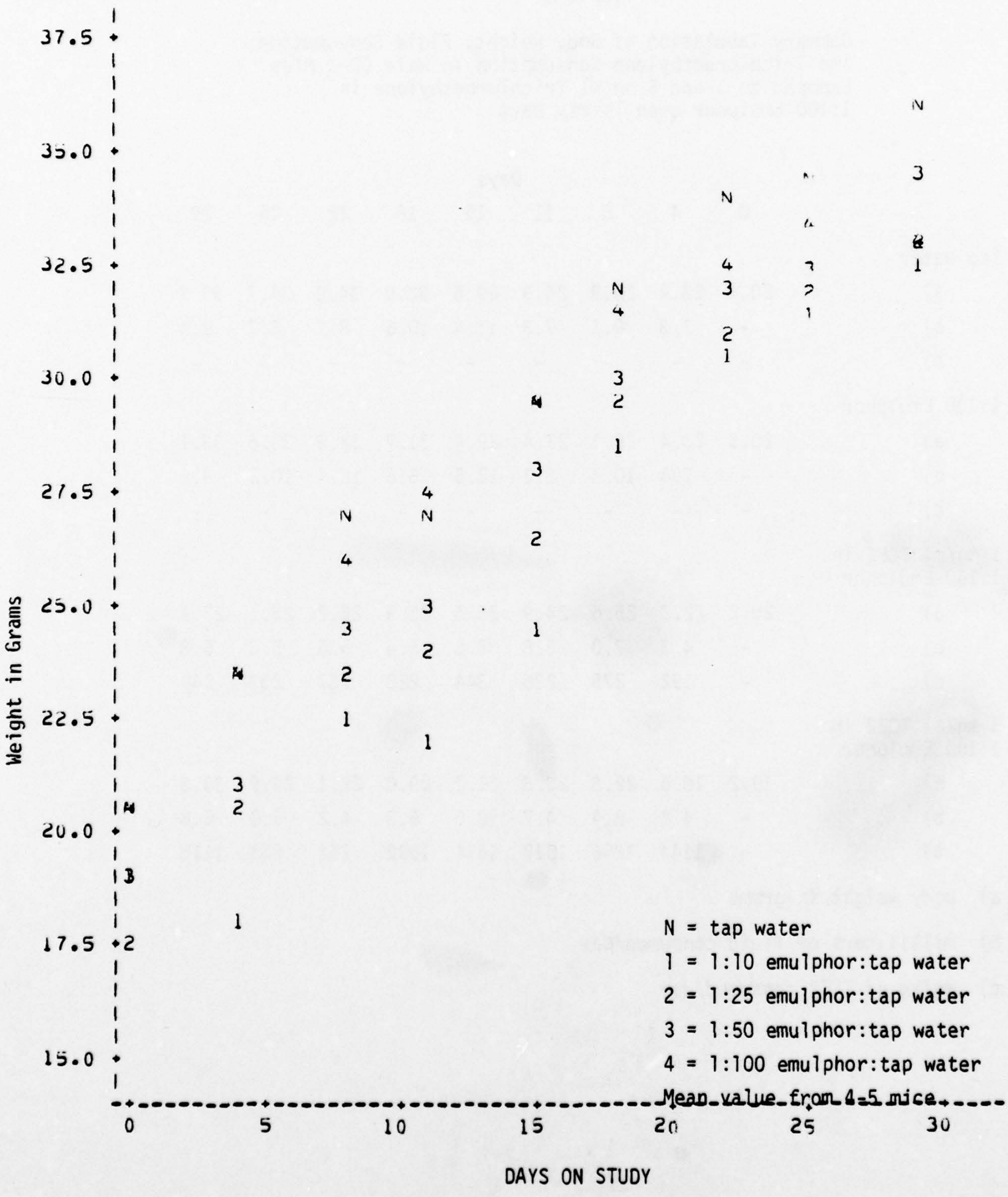


Figure 2

Body Weight Gain in CD-1 Male Mice

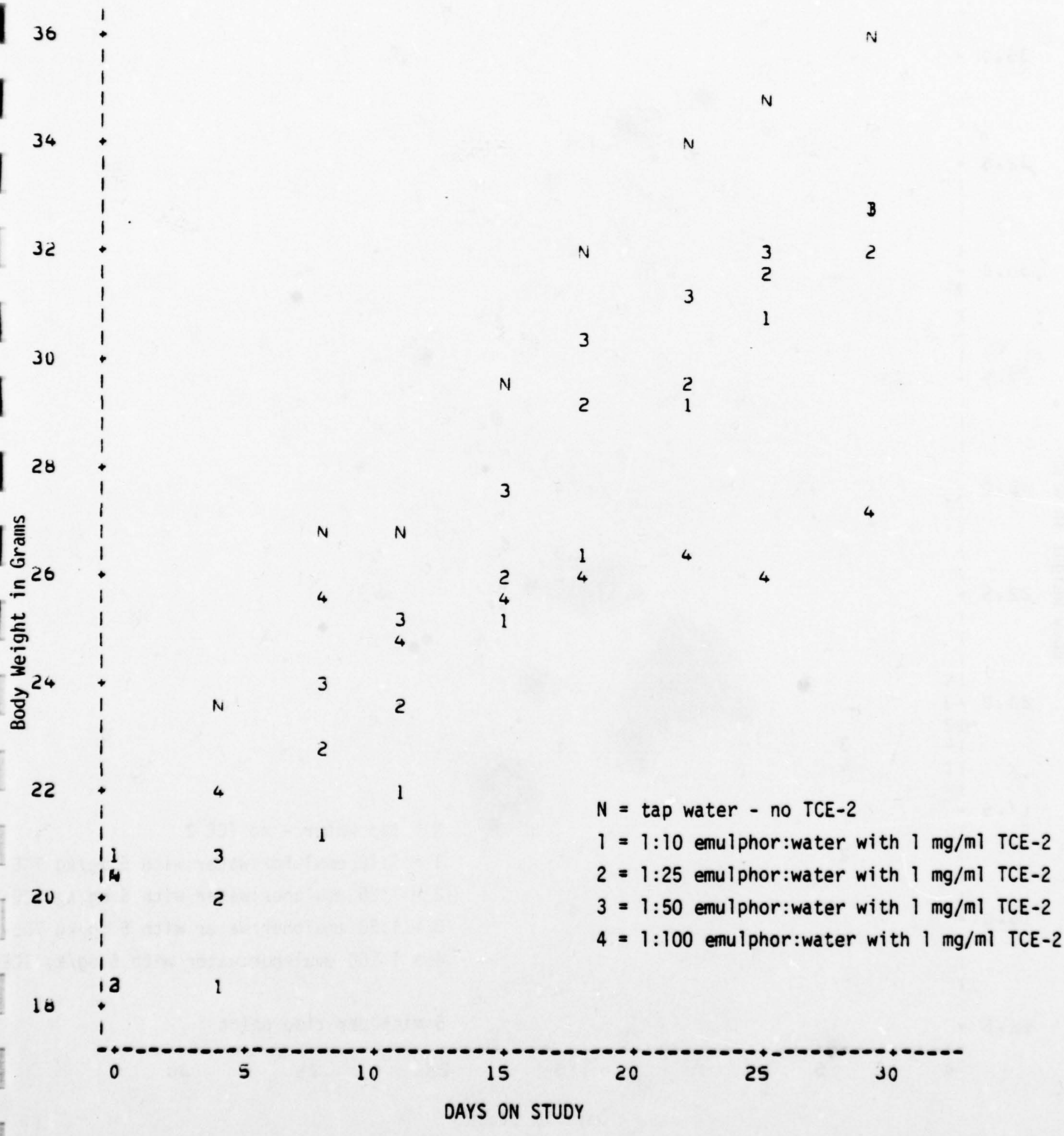


Figure 3
Body Weight Gain in CD-1 Male Mice

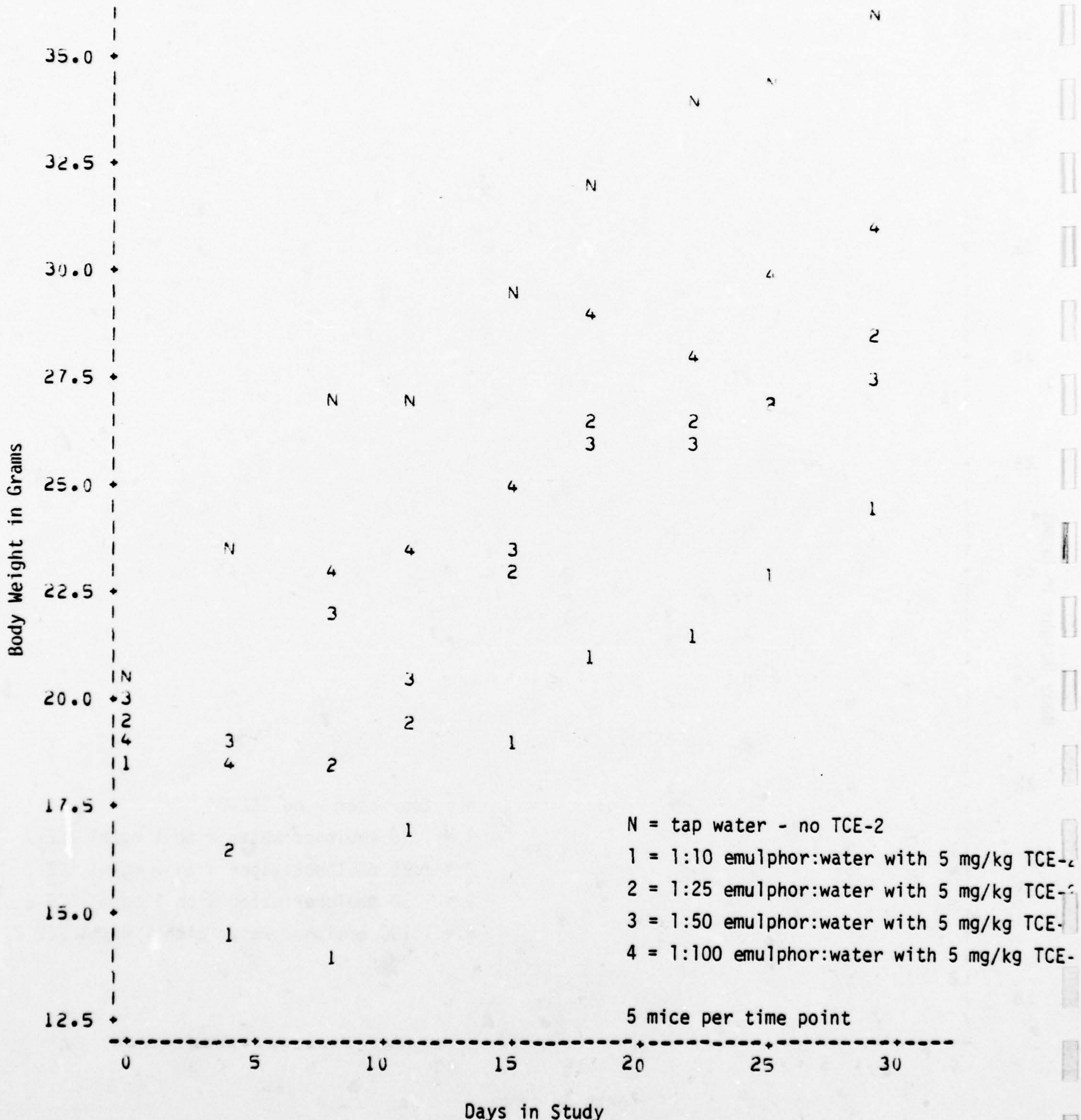


Figure 4
Body Weight Gain in CD-1 Male Mice

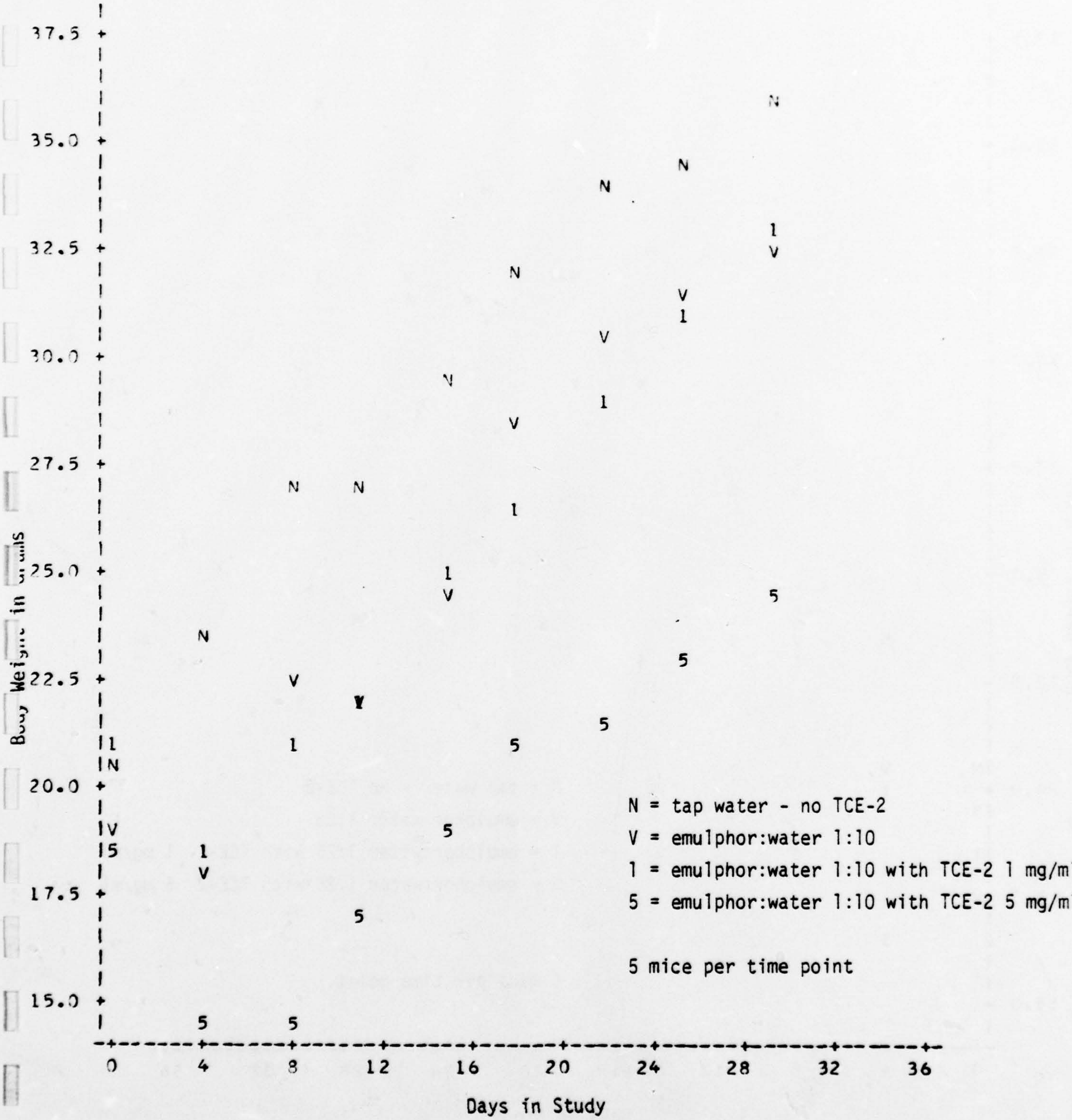


Figure 5
Body Weight Gain in CD-1 Male Mice

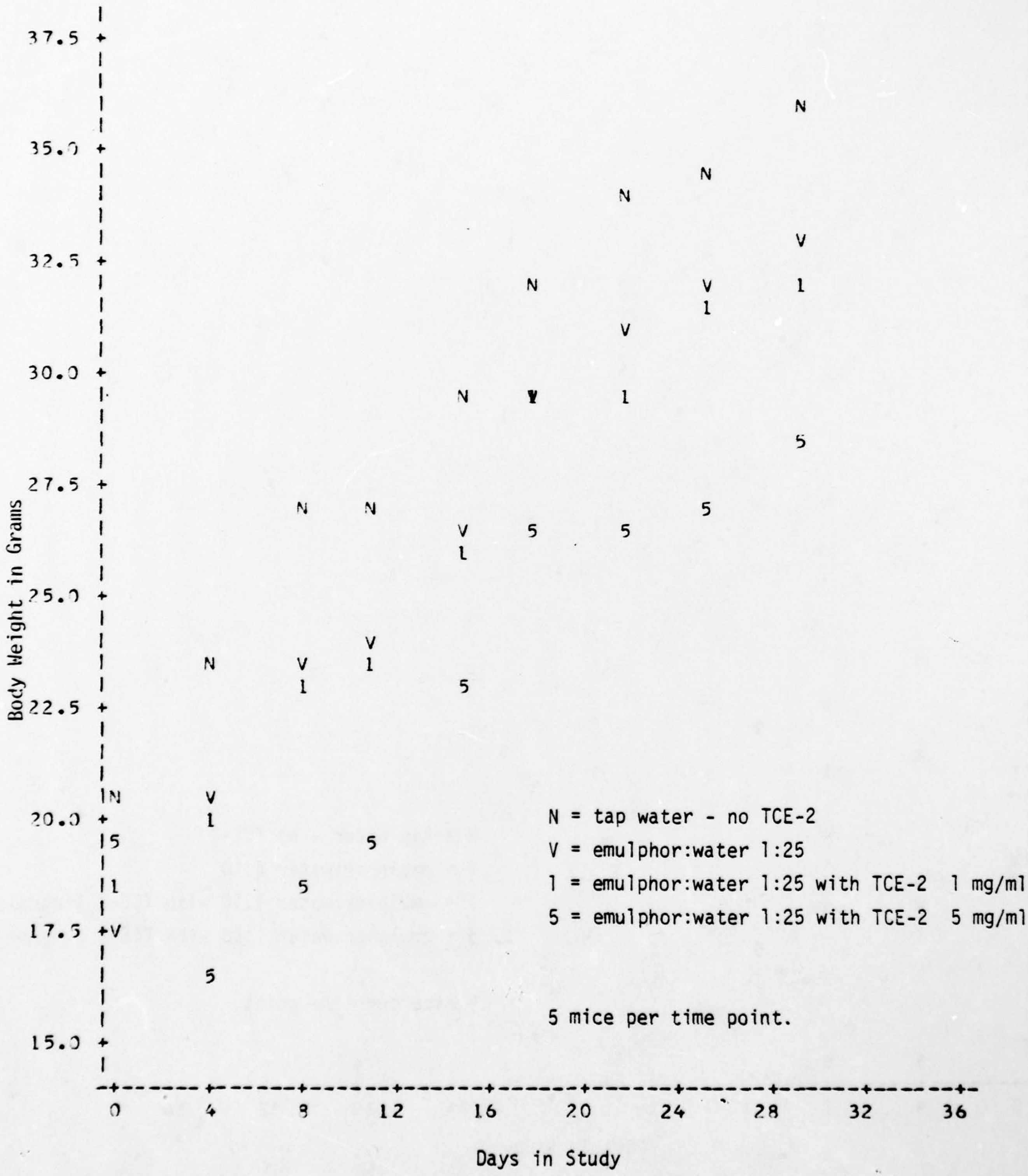


Figure 6
Body Weight Gain in CD-1 Male Mice

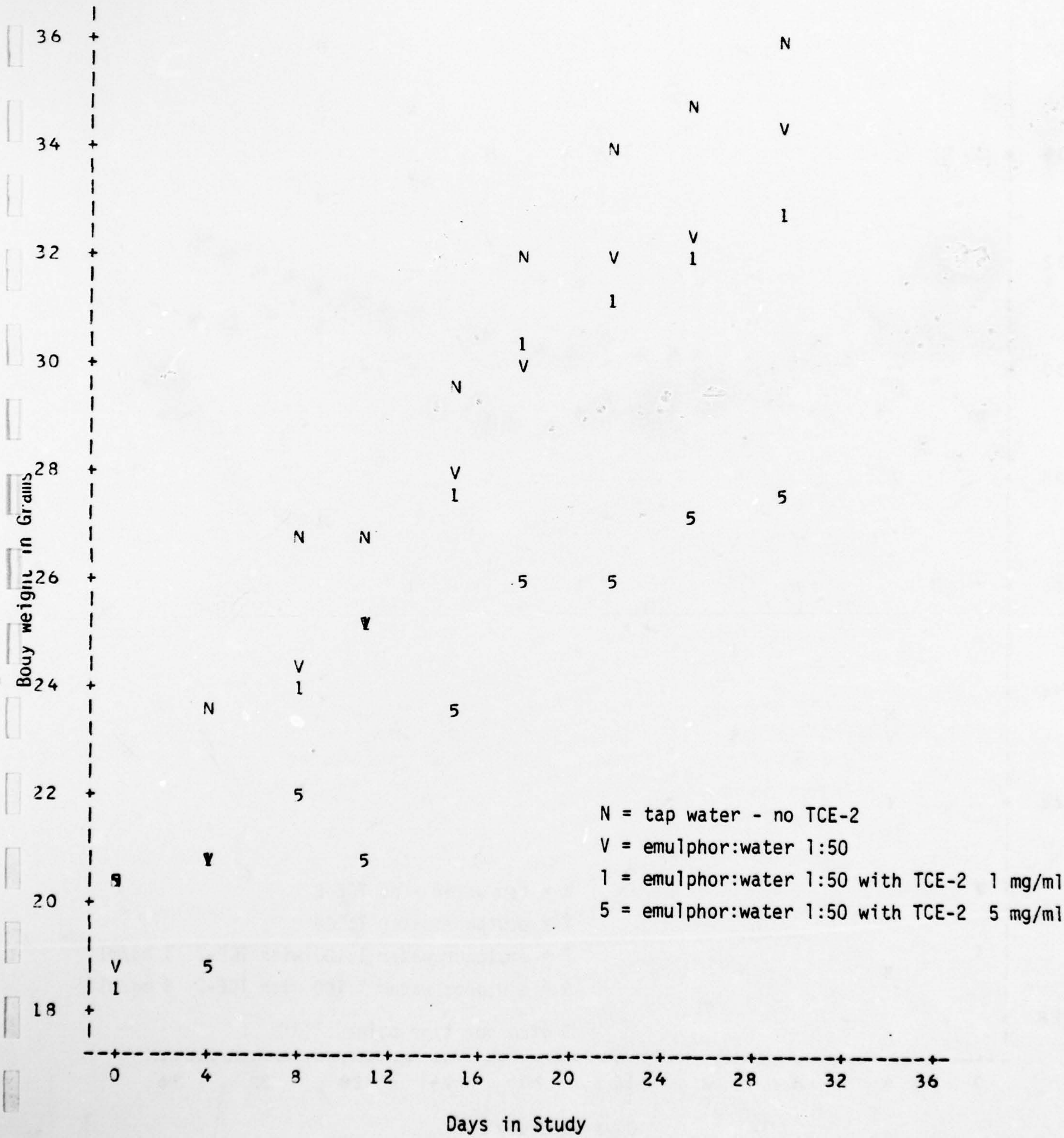
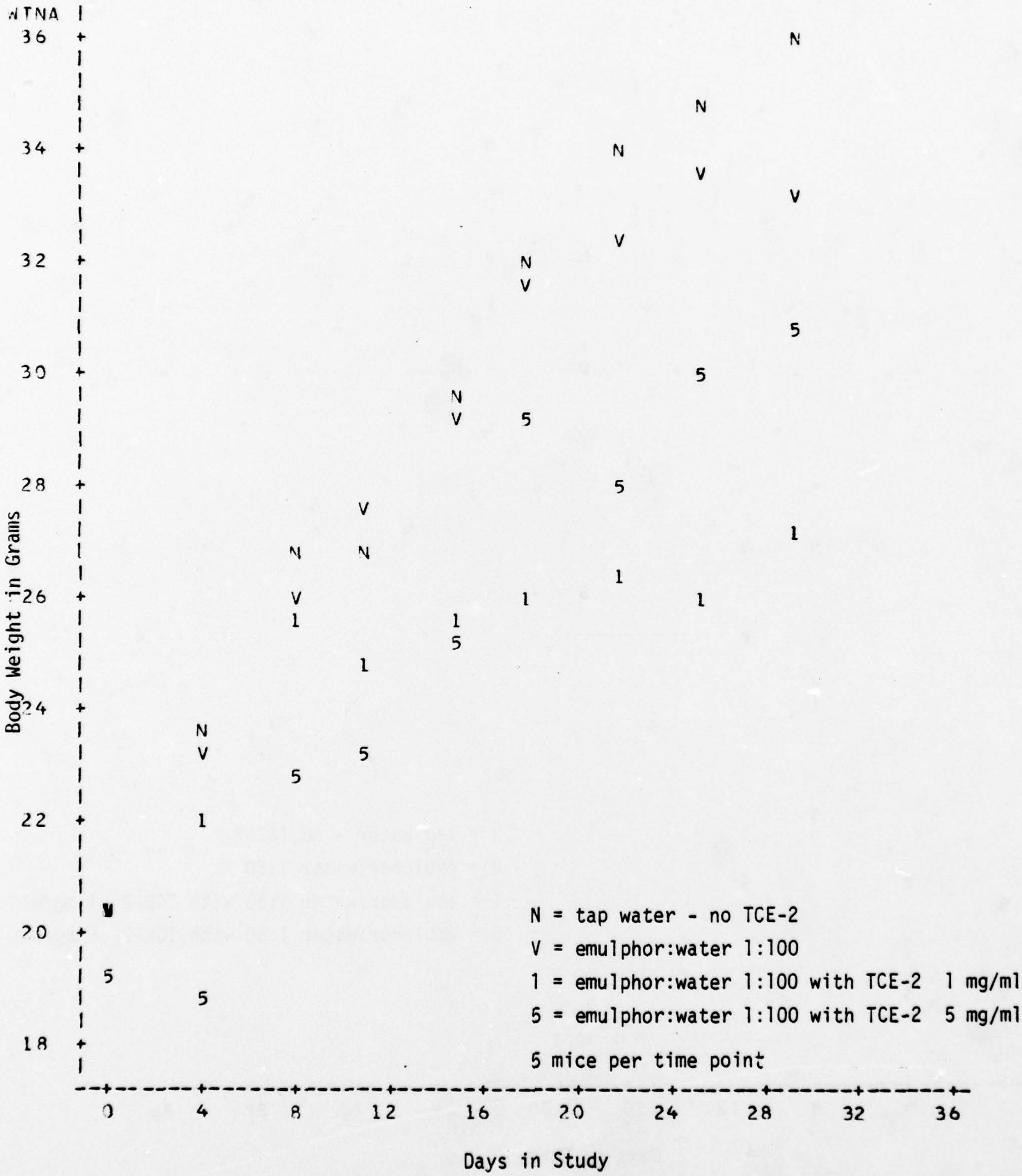


Figure 7
Body Weight Gain in CD-1 Male Mice



amount of chemical. However, the lowest concentration tested, 1% emulphor, shows the least effects on fluid consumption and body weight gain. The calculated doses of trichloroethylene in mg/kg per day as a function of emulphor concentration are seen in Table 9. The mg/kg of TCE-2 per day consumed at the 1 mg/kg TCE-2 concentration were 217, 268, 304, 248 mg/kg for the 10%, 4%, 2%, and 1% emulphor concentrations, respectively. In the case of 5 mg/ml of TCE-2, the mg/kg per day consumed was indicated earlier. More information on the delivery of TCE-2 in emulphor is provided in the subchronic study of TCE-2.

On the 31st day of the study, one of the two cages from each of the experimental groups was used to measure a number of the toxicological variables. Since we were looking for marked effects and this represented a pilot study, we used a very small number of mice per group. The results of the toxicological responses are summarized below. (Statistical analysis was done using both the one-way and the two-way analysis of variance.)

Organ weights were expressed in three ways: in milligrams, as a percentage of the whole body weight, and as a ratio of the organ weight to the brain weight. Table 10 shows the data on brain weight in mg and as percent of body weight. There were no significant alterations in brain weight as a function of emulphor concentration or TCE-2 concentration.

Liver weights were increased in mice exposed to 5 mg/ml of TCE-2 in the 4% emulphor when expressed in mg or as liver-to-brain ratio (Table 11). When expressed as percent body weight, the liver weights were increased in the three highest vehicle concentrations in the 5 mg/ml TCE-2. An increase of 34% was also seen in mice dosed with 1 mg/ml TCE-2 in 10% emulphor.

Emulphor produced a concentration-dependent reduction in spleen weight (Table 12). This was statistically significant in the 4 and 10% emulphor groups when calculated in mg and as percent of body weight. Significance in spleen weight reduction when computed as a ratio of brain weight was seen at the 10% emulphor concentration. There was no discernible TCE-2 effect on the spleen weight.

Lung weights were not altered by emulphor or by TCE-2 (Table 13).

Thymus weights, when expressed in mg or as thymus-to-brain ratio (Table 14), were significantly increased in mice exposed to 1 and 5 mg/ml of TCE-2 in the 1% emulphor. Thymus weights expressed as percent of body weight show the same trend. These changes appear to be caused by the small thymus weight in the 1% emulphor control group (Table 14).

Table 9

Calculated Doses of Trichloroethylene in mg/kg/day

Group	N	Emulphor:Water	Concentration of TCE-2	Mg/kg TCE-2/day Consumed
1	80	1:10	1	217 ± 6.5
2	80	1:25	1	268 ± 9.6
3	80	1:50	1	304 ± 8.4
4	80	1:100	1	248 ± 12.2
5	80	1:10	5	1004 ± 38
6	80	1:25	5	1067 ± 39
7	80	1:50	5	939 ± 31
8	80	1:100	5	1181 ± 45

Groups of CD-1 male mice were administered trichloroethylene in the drinking water in varying concentrations of emulphor: Trichloroethylene dose was calculated from the fluid consumption and the individual mouse weights. Data on mg/kg of TCE-2 is given as mean ± standard errors derived from 80 records.

Table 10

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Brain Weights in Weanling Male Mice[a]

	TCE (mg/ml)					
Emulphor:water	0		1		5	
	mg	% BW	mg	% BW	mg	% BW
0	427 ± 19 (5)[b]	1.19 ± 0.07 (5)	-	-	-	-
1:10	418 ± 6 (4)	1.29 ± 0.04 (4)	431 ± 10 (5)	1.19 ± 0.05 (5)	415 ± 8 (4)	1.55 ± 0.13 (4)
1:25	444 ± 16 (4)	1.37 ± 0.09 (4)	431 ± 3 (5)	1.33 ± 0.04 (5)	400 ± 19 (5)	1.41 ± 0.10 (5)
1:50	421 ± 23 (5)	1.20 ± 0.07 (5)	441 ± 19 (5)	1.28 ± 0.07 (5)	390 ± 19 (5)	1.32 ± 0.07 (5)
1:100	397 ± 6 (5)	1.16 ± 0.05 (5)	444 ± 7 (5)	1.27 ± 0.08 (5)	402 ± 8 (5)	1.21 ± 0.06 (5)

NOTE: Where brain weights are expressed in milligrams (mg), for Vehicle (effect due to emulphor concentration), $p < 0.63$; for TCE (effect due to trichloroethylene) $p < 0.005$; for Vehicle + TCE (effect due to interaction between emulphor and trichloroethylene), $p < 0.38$. Where brain weights are expressed as percent of body weight, for Vehicle, $p < 0.05$; for TCE, $p < 0.07$, for Vehicle + TCE, $p < 0.18$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

Table 11

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Liver Weights in Weanling Male Mice[a]

Emulphor:water	TCE (mg/ml) 0			1			5		
	mg	% BW	Liver/Brain	mg	% BW	Liver/Brain	mg	% BW	Liver/Brain
0	962 ± 177 (5)[b]	5.43 ± .48 (5)	4.61 ± .40 (5)	-	-	-	-	-	-
1:10	2246 ± 184 (4)	6.93 ± .53 (4)	5.37 ± .42 (4)	3303 ± 95[c] (5)	9.12 ± .45[c] (5)	7.67 ± .16[c] (5)	2402 ± 184 (4)	8.88 ± .61[c] (4)	5.80 ± .43 (4)
1:25	1947 ± 107 (4)	6.05 ± .48 (4)	4.41 ± .31 (4)	2233 ± 99 (5)	6.93 ± .32 (5)	5.19 ± .25 (5)	2687 ± 114[c] (5)	9.28 ± .64[c] (5)	6.59 ± .32[c] (5)
1:50	2061 ± 52 (5)	5.90 ± .18 (5)	4.93 ± .23 (5)	2162 ± 102 (5)	6.13 ± .21 (5)	4.87 ± .38 (5)	2154 ± 84 (5)	7.28 ± .13[c] (5)	5.58 ± .35 (5)
1:100	2014 ± 121 (5)	5.86 ± .15 (5)	5.08 ± .35 (5)	2214 ± 223 (5)	6.21 ± .40 (5)	5.01 ± .56 (5)	2289 ± 168 (5)	6.83 ± .40 (5)	5.73 ± .52 (5)

NOTE: Where liver weights are expressed in milligrams (mg) for Vehicle (effect due to emulphor concentration), $p < 0.0001$; for TCE (effect due to trichloroethylene), $p < 0.0006$; and for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.0003$. Where liver weights are expressed as percent of body weight (% BW), for Vehicle, $p < 0.0001$; TCE, $p < 0.0001$; Vehicle + TCE, $p < 0.02$. Where liver weights are expressed as liver-to-brain ratio (Liver/Brain), for Vehicle, $p < 0.003$; TCE, $p < 0.003$, Vehicle + TCE, $p < 0.001$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

[c]TCE significantly different from respective vehicle groups at $p < 0.05$ as determined by Dunnett's T Test.

Table 12

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Spleen Weights in Weanling Male Mice[a]

	TCE (mg/ml)								
	0			1			5		
Emulphor:water	mg	% BW	Spleen/Brain	mg	% BW	Spleen/Brain	mg	% BW	Spleen/Brain
0	253 ± 16.1 (5)[b]	.70 ± .06 (5)	.59 ± .03 (5)	-	-	-	-	-	-
1:10	145 ± 19.2[c] (4)	.45 ± .05[c] (4)	.35 ± .05[c] (4)	162 ± 11.8 (5)	.45 ± .03 (5)	.38 ± .02 (5)	115 ± 8.8 (4)	.43 ± .03 (4)	.28 ± .02 (4)
1:25	178 ± 11.0[c] (4)	.55 ± .048[c] (4)	.40 ± .02 (4)	193 ± 18.6 (5)	.60 ± .07 (5)	.45 ± .04 (5)	181 ± 10.0 (5)	.62 ± .03 (5)	.44 ± .02 (5)
1:50	192 ± 21.4 (5)	.55 ± .06 (5)	.47 ± .07 (5)	195 ± 18.6 (5)	.56 ± .04 (5)	.45 ± .05 (5)	188 ± 25.9 (5)	.63 ± .07 (5)	.48 ± .05 (5)
1:100	240 ± 22.9 (5)	.70 ± .05 (5)	.61 ± .06 (5)	319 ± 31.3 (5)	.92 ± .13 (5)	.72 ± .07 (5)	190 ± 22.1 (5)	.57 ± .07 (5)	.47 ± .05 (5)

NOTE: Where spleen weights are expressed in milligrams (mg), for Vehicle (effect due to emulphor concentration), $p < 0.0001$; for TCE (effect due to trichloroethylene), $p < 0.004$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.06$. Where spleen weights are expressed as percent of body weight (% BW), for Vehicle, $p < 0.0001$; for TCE, $p < 0.21$; for Vehicle + TCE, $p < 0.05$. Where spleen weights are expressed as spleen-to-brain ratio (Spleen/Brain), for Vehicle, $p < 0.0001$; for TCE, $p < 0.054$; for Vehicle + TCE, $p < 0.084$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

[c]Vehicle significantly different from naive group at $p < 0.05$ as determined by Dunnett's T Test.

Table 13

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Lung Weights in Weanling Male Mice[a]

Emulphor:water	TCE (mg/ml) 0			1			5		
	mg	% BW	Lung/Brain	mg	% BW	Lung/Brain	mg	% BW	Lung/Brain
0	252 ± 12.6 (5)[b]	.70 ± .03 (5)	.60 ± .05 (5)	-	-	-	-	-	-
1:10	204 ± 6.9 (4)	.63 ± .03 (4)	.49 ± .02 (4)	210 ± 8.5 (5)	.58 ± .03 (5)	.49 ± .02 (5)	208 ± 16.5 (4)	.78 ± .09 (4)	.50 ± .04 (4)
1:25	209 ± 14.2 (4)	.65 ± .05 (4)	.47 ± .02 (4)	213 ± 11.9 (5)	.66 ± .02 (5)	.50 ± .03 (5)	226 ± 13.9 (5)	.77 ± .02[c] (5)	.55 ± .04 (5)
1:50	239 ± 3.7 (5)	.68 ± .02 (5)	.57 ± .04 (5)	232 ± 11.4 (5)	.68 ± .05 (5)	.53 ± .01 (5)	213 ± 21.6 (5)	.72 ± .05 (5)	.54 ± .03 (5)
1:100	236 ± 19.0 (5)	.69 ± .04 (5)	.60 ± .05 (5)	250 ± 16.9 (5)	.71 ± .03 (5)	.57 ± .05 (5)	224 ± 6.3 (5)	.67 ± .03 (5)	.56 ± .02 (5)

NOTE: Where lung weights are expressed in milligrams (mg), for Vehicle (effect due to emulphor concentration), $p < 0.06$; for TCE (effect due to trichloroethylene), $p < 0.7$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.7$. Where lung weights are expressed as percent of body weight (% BW), Vehicle, $p < 0.8$; TCE, $p < 0.01$; Vehicle + TCE, $p < 0.1$. Where lung weights are expressed as lung-to-brain ratio (Lungs/Brain), Vehicle, $p < 0.01$; TCE, $p < 0.7$; Vehicle + TCE, $p < 0.6$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

[c]TCE significantly different from respective vehicle group at $p < 0.05$ as determined by Dunnett's T Test.

Table 14

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Thymus Weights in Weanling Male Mice[a]

Emulphor:water	TCE (mg/ml) 0 mg	0 % BW	0 Thymus/Brain	1 mg	1 % BW	1 Thymus/Brain	5 mg	5 % BW	5 Thymus/Brain
0	50.0 ± 5.7 (5)[b]	.14 ± .02 (5)	.12 ± .02 (5)	-	-	-	-	-	-
1:10	50.5 ± 4.2 (4)	.16 ± .01 (4)	.12 ± .01 (4)	40.6 ± 6.3 (5)	.11 ± .02 (5)	.09 ± .01 (5)	62.5 ± 2.6 (4)	.23 ± .01[c] (4)	.15 ± .01 (4)
1:25	62.5 ± 8.5 (4)	.20 ± .03 (4)	.14 ± .01 (4)	61.2 ± 4.3 (5)	.19 ± .01 (5)	.14 ± .01 (5)	65.2 ± 6.4 (5)	.22 ± .02 (5)	.16 ± .01 (5)
1:50	73.0 ± 14.7 (5)	.21 ± .04 (5)	.18 ± .04 (5)	60.6 ± 7.0 (5)	.18 ± .02 (5)	.14 ± .01 (5)	53.4 ± 9.8 (5)	.18 ± .03 (5)	.13 ± .02 (5)
1:100	40.5 ± 3.9 (5)	.12 ± .02 (5)	.10 ± .01 (5)	69.2 ± 7.0[c] (5)	.20 ± .01[c] (5)	.16 ± .02[c] (5)	62.4 ± 4.6[c] (5)	.19 ± .02[c] (5)	.16 ± .01[c] (5)

NOTE: Where thymus weights are expressed in milligrams (mg), for Vehicle (effect due to emulphor concentration), $p < 0.2$; for TCE (effect due to trichloroethylene), $p < 0.7$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.2$. Where thymus weights are expressed as percent of body weight (% BW), for Vehicle, $p < 0.2$; for TCE, $p < 0.7$; for Vehicle + TCE, $p < 0.02$. Where thymus weights are expressed as thymus-to-brain ratio, for Vehicle, $p < 0.2$; for TCE, $p < 0.7$; for Vehicle + TCE, $p < 0.02$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

[c]TCE significantly different from respective vehicle group at $p < 0.05$ as determined by Dunnett's T Test.

Kidney weights were increased when expressed in mg and as kidney-to-brain ratio in mice exposed to 1 mg/ml of TCE-2 in the 10% emulphor. However, at 5 mg/ml of TCE-2, the kidney weights decreased. These changes show no trend, and no interpretation on the interaction of emulphor and TCE-2 on kidney weight can be made (Table 15).

The testes weights in these animals are shown in Table 16. No biologically significant changes were seen.

Leucocyte counts (Table 17) in mice exposed to the 1% emulphor are increased 77% above tap water controls. Compared to mice exposed to 1% emulphor, TCE-2 exposed mice in both the 1 and 5 mg/kg groups have significantly lower leucocyte counts. This does not represent the TCE-2 effect, since the values are within the control range for not exposed to TCE-2. Platelet counts were not altered by emulphor or TCE-2.

Erythrocyte counts (Table 18) were depressed by 39% in mice exposed to 4% emulphor as compared to mice exposed to tap water. RBC counts in mice exposed to TCE-2 were elevated above the appropriate vehicle control for both the 1 and 5 mg/ml TCE-2 in the 4% and 2% emulphor. Erythrocyte counts were depressed 15% in mice exposed to 5 mg/ml in the 1% vehicle. These changes are probably not related to TCE-2 since there is no apparent trend. No significant changes were observed in hemoglobin or hematocrit values.

Serum glutamic pyruvate transaminase and blood urea nitrogen levels (Table 19) were also within vehicle control values. Although there are sporadic statistical differences, these values are within control range.

Selected parameters were measured for blood coagulation. Fibrinogen levels were elevated in all mice exposed to 1% emulphor, with or without TCE-2. Fibrinogen in 1% emulphor by itself was increased 32% above tap water control. Mice exposed to 5 mg/ml of TCE-2 in 4% emulphor and 1 mg/ml in the 2% emulphor also had elevated fibrinogen levels as compared to control (Table 20). Activated partial thromboplastin times were increased in mice exposed to 5 mg/ml in the 4% and 1% emulphor. Prothrombin times were increased at the 1 and 5 mg/ml in the 10% vehicle and at 5 mg/ml in the 4% and 2% vehicle.

Table 15

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Kidney Weights in Weanling Male Mice[a]

Emulphor:water	TCE (mg/ml) 0			1			5		
	mg	% BW	Kidney/Brain	mg	% BW	Kidney/Brain	mg	% BW	Kidney/Brain
0	637 ± 21.1 (5)[b]	1.77 ± .09 (5)	1.50 ± .05 (5)	-	-	-	-	-	-
1:10	533 ± 14.9[c] (4)	1.65 ± .07 (4)	1.27 ± .02 (4)	640 ± 28.1[d] (5)	1.77 ± .12 (5)	1.48 ± .04[d] (5)	452 ± 13.0[d] (4)	1.68 ± .11 (4)	1.09 ± .04[d] (4)
1:25	553 ± 29.4 (4)	1.72 ± .13 (4)	1.25 ± .09[c] (4)	580 ± 27.0 (5)	1.80 ± .06 (5)	1.35 ± .07 (5)	543 ± 32.8 (5)	1.85 ± .05 (5)	1.33 ± .06 (5)
1:50	650 ± 19.4 (5)	1.86 ± .05 (5)	1.55 ± .05 (5)	638 ± 12.3 (5)	1.85 ± .07 (5)	1.45 ± .05 (5)	493 ± 25.1[d] (5)	1.66 ± .04 (5)	1.27 ± .08 (5)
1:100	549 ± 29.7 (5)	1.60 ± .05 (5)	1.38 ± .07 (5)	590 ± 20.3 (5)	1.69 ± .09 (5)	1.33 ± .06 (5)	588 ± 25.8 (5)	1.76 ± .03 (5)	1.47 ± .08 (5)

NOTE: Where kidney weights are expressed as milligrams (mg), for Vehicle (effect due to emulphor concentration), $p < 0.08$; for TCE (effect due to trichloroethylene), $p < 0.0001$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.0007$. Where kidney weights are expressed as percent of body weight (% BW), for Vehicle, $p < 0.2$; for TCE, $p < 0.5$; for Vehicle + TCE, $p < 0.3$. Where kidney weights are expressed as kidney-to-brain ratio (Kidney/Brain), for Vehicle, $p < 0.03$; for TCE, $p < 0.04$; for Vehicle + TCE, $p < 0.002$.

[a] Values represent mean ± standard error.

[b] ( ) = number of mice/group.

[c] Vehicle significantly different from naive group at $p < 0.05$ as determined by Dunnett's T Test.

[d] TCE significantly different from respective vehicle groups at $p < 0.05$ as determined by Dunnett's T Test.

Table 16

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Testes Weights in Weanling Male Mice[a]

Emulphor:water	TCE (mg/ml) 0			1			5		
	mg	% BW	Testes/Brain	mg	% BW	Testes/Brain	mg	% BW	Testes/Brain
0	239 ± 15 (5)[b]	.66 ± .04 (5)	.57 ± .06 (5)	-	-	-	-	-	-
1:10	234 ± 18.0 (4)	.73 ± .06 (4)	.56 ± .04 (4)	227 ± 6.3 (5)	.63 ± .03 (5)	.53 ± .02 (5)	226 ± 13.8 (4)	.84 ± .07 (4)	.55 ± .04 (4)
1:25	241 ± 16.0 (4)	.75 ± .06 (4)	.55 ± .04 (4)	220 ± 16.6 (5)	.68 ± .06 (5)	.51 ± .04 (5)	247 ± 14.5 (5)	.85 ± .06 (5)	.60 ± .01 (5)
1:50	259 ± 11.2 (5)	.74 ± .02 (5)	.62 ± .04 (5)	247 ± 8.4 (5)	.72 ± .03 (5)	.56 ± .02 (5)	213 ± 15.8[c] (5)	.72 ± .05 (5)	.54 ± .01 (5)
1:100	233 ± 12.8 (5)	.68 ± .03 (5)	.59 ± .03 (5)	261 ± 12.3 (5)	.75 ± .06 (5)	.59 ± .03 (5)	252 ± 12.3 (5)	.75 ± .04 (5)	.63 ± .03 (5)

NOTE: Where testes weights are expressed in milligrams (mg), for Vehicle (effect of emulphor concentration), $p < 0.4$; for TCE (effect due to trichloroethylene), $p < 0.7$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.1$. Where testes weights are expressed as percent of body weight (% BW), for Vehicle, $p < 0.08$; for TCE, $p < 0.02$; for Vehicle + TCE, $p < 0.2$. Where testes weights are expressed as testes-to-brain ratio (Testes/Brain), for Vehicle, $p < 0.1$; for TCE, $p < 0.2$; for Vehicle + TCE, $p < 0.3$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

[c]TCE significantly different from respective vehicle group at $p < 0.05$ as determined by Dunnett's T Test.

Table 17

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Leucocyte and Platelet Counts in Weanling Male Mice[a]

	TCE (mg/ml)					
Emulphor:water	Leucocyte Counts ($x10^3/mm^3$)			Platelet Counts ($x10^5/mm^3$)		
	0	1	5	0	1	5
0	12.14 ± 1.24 (5)[b]	-	-	2.14 ± 0.25 (5)	-	-
1:10	10.35 ± 2.10 (4)	11.52 ± 1.40 (5)	9.48 ± 1.87 (4)	2.04 ± 0.15 (4)	1.89 ± 0.22 (5)	1.60 ± 0.11 (4)
1:25	12.68 ± 1.84 (4)	11.48 ± 0.92 (5)	12.30 ± 1.02 (5)	1.89 ± 0.17 (4)	2.17 ± 0.20 (5)	1.97 ± 0.08 (5)
1:50	13.28 ± 0.80 (5)	13.62 ± 0.48 (5)	16.98 ± 2.29 (5)	1.57 ± 0.09 (5)	1.71 ± 0.18 (5)	1.82 ± 0.14 (5)
1:100	21.45 ± 2.19[c] (5)	13.48 ± 1.45[d] (5)	14.26 ± 0.93[d] (5)	1.52 ± 0.20 (5)	1.47 ± 0.16 (5)	1.77 ± 0.09 (5)

NOTE: Where the values represent leucocyte counts, for Vehicle (effect due to emulphor concentration), $p < 0.0001$; for TCE (effect due to trichloroethylene), $p < 0.2$, for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.01$. Where values represent platelet counts, for Vehicle, $p < 0.01$; for TCE, $p < 0.9$; for Vehicle + TCE, $p < 0.2$.

[a] Values represent mean ± standard error.

[b] ( ) = number of mice/group.

[c] Significantly different from naive group at $p < 0.05$ as determined by Dunnett's T Test.

[d] TCE significantly different from respective vehicle group at $p < 0.05$ as determined by Dunnett's T Test.

Table 18

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Erythrocyte Counts, Hemoglobin Values, and Hematocrit Values in Weanling Male Mice[a]

	TCE (mg/ml)								
Emulphor:water	Erythrocyte Counts ($x10^6/mm^3$)			Hemoglobin (g%)			Hematocrit (%)		
	0	1	5	0	1	5	0	1	5
0	7.12 ± .29 (5)[b]	-	-	7.88 ± .19 (5)	-	-	43.0 ± .55 (5)	-	-
1:10	6.45 ±1.02 (4)	6.39 ±.07 (5)	6.92 ± .39 (4)	8.40 ± .49 (4)	7.76 ± .16 (5)	7.93 ± .13 (4)	42.8 ± .85 (4)	42.2 ± .73 (5)	41.3 ± .63 (4)
1:25	4.96 ± .60[c] (4)	6.94 ±.17[d] (5)	6.80 ± .25[d] (5)	8.85 ± .78 (4)	7.76 ± .30 (5)	7.42 ± .42 (5)	41.8 ± .87 (4)	41.2 ± .58 (5)	43.4 ± 1.03 (5)
1:50	5.31 ± .45 (5)	7.43 ±.25[d] (5)	6.89 ± .10[d] (5)	7.88 ± .43 (5)	8.86 ± .50 (5)	7.73 ± .35 (5)	43.5 ± 1.5 (5)	42.6 ± .87 (5)	42.8 ± .25 (5)
1:100	7.42 ± .27 (5)	7.66 ± .38 (5)	6.35 ± .12[d] (5)	8.48 ± .63 (5)	7.66 ± .37 (5)	8.32 ± .59 (5)	45.3 ± .63 (5)	41.6 ± .68[c] (5)	46.4 ± .40 (5)

NOTE: Where values represent erythrocyte counts, for Vehicle (effect due to emulphor concentration), $p < 0.04$; for TCE (effect due to trichloroethylene), $p < 0.001$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.003$. Where values represent hemoglobin, for Vehicle, $p < 0.97$; for TCE, $p < 0.25$; for Vehicle + TCE, $p < 0.21$. Where values represent hematocrit, for Vehicle, $p < 0.0002$; for TCE, $p < 0.014$; for Vehicle + TCE, $p < 0.014$.

[a]Values represent mean ± standard error.

[b]( ) = number of mice/group.

[c]Vehicle significantly different from naive group at $p < 0.05$ as determined by Dunnett's T Test.

Table 19

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Serum Glutamic Pyruvic Transaminase and Blood Urea Nitrogen Levels in Weanling Male Mice[a]

	TCE (mg/ml)					
Emulphor:water	SGPT (units/ml)			BUN (mg/dl)		
	0	1	5	0	1	5
0	22.0 ± 7.0 (5)[b]	-	-	23.8 ± 1.8 (5)	-	-
1:10	21.5 ± 2.2 (4)	24.8 ± 8.4 (5)	11.5 ± 2.2 (4)	28.6 ± 2.7 (4)	25.3 ± 0.9 (5)	27.3 ± 0.7 (4)
1:25	12.0 ± 1.2 (4)	12.4 ± 0.9 (5)	12.0 ± 1.3 (5)	25.3 ± 1.2 (4)	24.5 ± 1.2 (5)	26.0 ± 2.4 (5)
1:50	22.0 ± 3.3 (5)	10.6 ± 2.4[c] (5)	17.6 ± 2.0 (5)	26.5 ± 1.6 (5)	25.1 ± 1.9 (5)	30.7 ± 1.3[c] (5)
1:100	30.0 ± 4.1 (5)	21.2 ± 4.8 (5)	16.0 ± 2.4 (5)	25.9 ± 1.5 (5)	27.7 ± 1.5 (5)	28.4 ± 1.2 (5)

NOTE: Where values represent SGPT, for Vehicle (effect due to emulphor concentration), $p < 0.01$; for TCE (effect due to trichloroethylene), $p < 0.04$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.09$. Where values represent BUN, for Vehicle, $p < 0.3$; for TCE, $p < 0.09$; for Vehicle + TCE, $p < 0.5$.

[a] Values represent mean ± standard error.

[b] ( ) = number of mice/group.

[c] TCE significantly different from respective vehicle group at $p < 0.05$ as determined by Dunnett's T Test.

Table 20

Effect of Thirty Day Exposure to Trichloroethylene and Varying Amounts of Emulphor in Drinking Water on Fibrinogen Levels, Activated Partial Thromboplastin Time, and Prothrombin Time in Weanling Male Mice[a]

	TCE (mg/ml)								
Emulphor:water	Fibrinogen (mg/dl)			APTT (sec)			Prothrombin Time (sec)		
	0	1	5	0	1	5	0	1	5
0	250.0 ± 10.4 (5)[b]	-	-	33.80 ± 4.12 (5)	-	-	7.60 ± .12 (5)	-	-
1:10	225.0 ± 6.1 (4)	231 ± 3.7 (5)	221.3 ± 17.1 (4)	32.55 ± 0.66 (4)	39.28 ± 3.32 (5)	40.43 ± 3.01 (4)	7.05 ± .14 (4)	8.30 ± .00[c] (5)	9.05 ± .14[c] (4)
1:25	217.5 ± 9.7 (4)	234 ± 6.0 (5)	269.0 ± 15.8[d] (5)	34.68 ± 1.82 (4)	34.04 ± 0.84 (5)	40.50 ± 0.98[c] (5)	7.43 ± .13 (4)	7.90 ± .19 (5)	8.40 ± .10[c] (5)
1:50	243.8 ± 15.5 (5)	290 ± 5.0[d] (5)	268.0 ± 11.8 (5)	36.05 ± 2.85 (5)	33.00 ± 0.97 (5)	38.00 ± 2.63 (5)	7.55 ± .25 (5)	8.00 ± .12 (5)	8.60 ± .37[c] (5)
1:100	331.3 ± 22.6[c] (5)	346 ± 6.0 (5)	342.0 ± 13.6 (5)	29.93 ± 1.03 (5)	34.50 ± 1.21 (5)	38.00 ± 1.35[c] (5)	8.30 ± .29 (5)	8.20 ± .19 (5)	8.40 ± .19 (5)

NOTE: Where values represent fibrinogen, for Vehicle (effect due to emulphor concentration), $p < 0.0001$; for TCE (effect due to trichloroethylene), $p < 0.03$; for Vehicle + TCE (effect due to an interaction between emulphor and trichloroethylene), $p < 0.1$. Where values represent APTT, for Vehicle, $p < 0.2$; for TCE, $p < 0.0004$; for Vehicle + TCE, $p < 0.2$. Where values represent prothrombin time, for Vehicle, $p < 0.1$; for TCE, $p < 0.0001$; for Vehicle + TCE, $p < 0.005$.

[a] Values represent mean ± standard error.

[b] ( ) = number of mice/group.

[c] Vehicle significantly different from naive group at $p < 0.05$ as determined by Dunnett's T Test.

[d] TCE significantly different from respective vehicle group at $p < 0.05$ as determined by Dunnett's T Test.

For reference, Table 21 provides historical control values over the last 6 months for untreated male mice (naive) and mice gavaged with 0.01 ml/gram of 10% emulphor in 0.15 M sodium chloride.

In summary, of the total of 31 toxicological parameters measured, 16 parameters were altered as a function of vehicle. No parameters were altered at the 1% dilution of emulphor:water. Seventeen parameters were specifically altered as a function of trichloroethylene and 13 parameters showed an interaction between trichloroethylene and emulphor.

Considering only the low concentrations of emulphor, i.e. 1%, and the high concentration of trichloroethylene, there were only four parameters significantly different from the appropriate control. These are thymus weight and activated partial thromboplastin time, which were elevated, and erythrocyte and leucocyte counts, which were decreased. In the case of leucocytes, the appropriate control group had an abnormally high leucocyte count compared to other controls. Although this data is derived from a pilot study with a small number of mice per group, it does suggest strongly that 1% emulphor has no significant effect on trichloroethylene. As will be seen in the subchronic study, 1% emulphor may cause some altered responses as compared to distilled water control.

Table 21
Historical Control Values
in CD-1 Male Mice

Response	Units	Naive	Vehicle	Combined
Hemoglobin	g %	11.4 ± 2.3 (74)	11.6 ± 2.6 (72)	11.5 ± 2.5 (146)
Hematocrit	%	36.8 ± 4.1 (62)	36.8 ± 4.8 (54)	36.7 ± 4.5 (119)
Leucocytes	$mm^3 \times 10^3$	8.5 ± 4.5 (65)	8.0 ± 3.6 (57)	8.3 ± 4.1 (122)
Platelets	$mm^3 \times 10^5$	2.3 ± 0.6 (62)	2.4 ± 0.9 (57)	2.4 ± 0.8 (119)
Erythrocytes	$mm^3 \times 10^6$	5.6 ± 1.3 (63)	5.4 ± 0.9 (55)	5.5 ± 1.2 (118)
Prothrombin Time	seconds	8.6 ± 1.1 (70)	8.6 ± 1.0 (74)	8.6 ± 1.1 (144)
APTT	seconds	33.6 ± 7.0 (46)	34.5 ± 7 (52)	34.0 ± 7.0 (98)
Fibrinogen	mg %	261 ± 75 (63)	246 ± 70 (68)	253 ± 73 (131)
SGPT	units/ml	22 ± 9 (69)	21 ± 10 (73)	22 ± 9 (142)
BUN	mg %	24 ± 5 (63)	23 ± 5 (60)	24 ± 5 (123)
Liver	% body weight liver/brain	5.5 ± 0.6 4.7 ± 0.8 (69)	6.1 ± 1.2 5.0 ± 1.0 (65)	5.8 ± 0.9 4.9 ± 0.9 (134)
Spleen	% body weight spleen/brain	0.53 ± 0.12 0.46 ± 0.11 (66)	0.52 ± 0.12 0.43 ± 0.10 (61)	0.53 ± 0.13 0.45 ± 0.11 (127)
Lungs	% body weight lungs/brain	0.71 ± 0.12 (69)	0.73 ± 0.17 (65)	0.72 ± 0.15 (134)

Response	Units	Naive	Vehicle	Combined
Thymus	% body weight	0.18 ± 0.06	0.19 ± 0.08	0.19 ± 0.08
	thymus/brain	0.16 ± 0.05	0.15 ± 0.07	0.16 ± 0.06
		(68)	(65)	(133)
Kidney	% body weight	1.62 ± 0.19	1.57 ± 0.27	1.59 ± 0.23
	kidney/brain	1.38 ± 0.22	1.30 ± 0.29	1.34 ± 0.26
		(68)	(64)	(132)
Testes	% body weight	0.65 ± 0.12	0.69 ± 0.14	0.67 ± 0.13
	testes/brain	0.56 ± 0.10	0.57 ± 0.12	0.57 ± 0.11
		(64)	(59)	(123)

Historical control values of 18 - 20 week old CD-1 mice. Naive = untreated. Vehicle = 10% emulphor in 0.15 M sodium chloride given in a volume of 0.01 mg/gram/day for 90 days. Numbers represent the mean ± standard deviation derived from the number of mice indicated in parenthesis.

Subchronic One-Hundred and Twenty Day Study on Trichloroethylene

Introduction

A four-month subchronic study of trichloroethylene administered in the drinking water was undertaken to construct a toxicologic data base in mice. This base is necessary for establishing the relevance of the *in vitro* responses in the tier assay system which is being developed. Additionally, trichloroethylene is of an environmental importance since it is widely distributed in our environment. On the basis of the trichloroethylene:emulphor interaction study previously discussed, we used a 1% emulphor solution to maintain the trichloroethylene in drinking water.

Animals and Housing

Male and female CD-1 mice, 15 to 20 grams, were obtained from Charles River Laboratories and quarantined for five days. The mice were housed five per cage on sawdust in plastic cages with wire tops and were identified by ear markings. Agway Laboratory Chow was used and provided *ad libitum* throughout the study period. The mice arrived at the Medical College of Virginia in 84 boxes of 26 animals per box and were placed in cages by the Central Animal Resources Facility in a randomized fashion. However, data indicates that this randomization was not complete.

A total of 450 cages were set up. One half of the mice were started on TCE-2 on February 9, 1979, and the remaining started on February 13, 1979. Six cages of vehicles and 3 cages from the other groups from each starting date were selected for weight monitoring and determination of fluid consumption. The weights of the mice at the end of the quarantine period for each group are shown in Table 22.

Materials and Methods

Trichloroethylene (TCE-2), certified ACS Lot 781557, was obtained from Fisher Scientific Co., Chemical Manufacting Division, Fairlawn, New Jersey 07410. TCE-2 has the following characteristics: chemical formula: $CHCl:CCl_2$; formula weight: 131.39; color: ALPHA-5; density: 1.456 grams/ml at 25° C; boiling point: 87.1° C ± 0.1°C; residue after evaporation: 0.002%; acidity (as HCl): 1 ppm, 0.0003%, water: 0.005%, heavy metals (as Pb): 0.02 ppm.

Trichloroethylene solutions were prepared twice weekly on Tuesday and Friday. The concentrations were 5, 2.5, 1, and 0.1 mg/ml in 1% emulphor (GAF EL620) as a 1% solution in distilled water. All preparations were maintained in dark bottles with cork stoppers. For stability and concentration monitoring, samples were taken from the initial TCE-2 preparations and at the times when the water bottles were changed. The samples were frozen and stored for analysis by gas chromatography.

Table 22

Body Weights of CD-1 Mice on the First Day of Exposure to Trichloroethylene

Group	Concentration (mg/ml)	N	2/9/79 Male	2/9/79 Female	2/13/79 Male	2/13/79 Female
Naive	—	15	25.6 ± 0.6	22.5 ± 0.4	20.8 ± 0.5	19.3 ± 0.3
Vehicle	—	30	23.2 ± 0.4	22.7 ± 0.3	25.7 ± 0.6	24.0 ± 0.3
TCE-2	0.1	15	25.2 ± 0.5	21.1 ± 0.3	29.2 ± 0.6	23.5 ± 0.2
TCE-2	1.0	15	25.0 ± 0.4	20.4 ± 0.4	27.7 ± 0.6	23.4 ± 0.6
TCE-2	2.5	15	25.3 ± 0.4	21.6 ± 0.4	29.0 ± 0.5	23.7 ± 0.3
TCE-2	5.0	15	24.6 ± 0.5	22.5 ± 0.5	26.6 ± 0.7	22.9 ± 0.4

Male and female mice were randomly placed into the cages (5 per cage). A total of 450 cages were set up: 225 males and 225 females. One half of the mice were started on TCE-2 on 2/9/79 and the remaining started on 2/13/79. Six cages of vehicle and 3 cages of each of the other groups were selected for weight monitoring. The numbers represent the mean ± standard error in grams.

After the quarantine period, groups of mice were exposed to the indicated concentrations of trichloroethylene. The fluid consumption was measured twice a week by weighing the water bottle, subtracting the difference between the weights, and dividing the grams consumed by the number of mice and the number of days. Body weights were determined twice weekly using a Mettler top-loading balance accurate to 0.1 grams. The variables which were monitored in addition to the body weight and food consumption were as follows:

1) Reference toxicologic responses:
   a) organ weights (brain, liver, spleen, lungs, thymus, kidneys, and testes)
   b) hematology (leucocyte, erythrocyte, and platelet counts, hemoglobin, and hematocrit)
   c) coagulation (fibrinogen levels and prothrombin time)
   d) hepatic mixed functional oxidase enzyme studies
      1) microsomal protein
      2) glutathione
      3) cytochrome P450
      4) aminopyrine demethylase
      5) analine hydroxylase
      6) cytochrome b_5
   e) bone marrow DNA synthesis, stem cell growth, and smears
2) Cell mediated immunity
   a) delayed type hypersensitivity to sheep erythrocytes (sRBC)
   b) spleen lymphocyte responsiveness to Concanovalin A
3) Humoral immunity
   a) spleen lymphocyte response to Lipopolysaccharide
   b) spleen IgM response to sRBC
4) Functional activity of the reticuloendothelial system
   a) vascular clearance of ^{51}Cr labelled sRBC
   b) recruitability of peritoneal cells
   c) phagocytosis of peritoneal adherent cells (macrophages)
   d) adherence of sheep erythrocytes to peritoneal cells (macrophages)
   e) chemotaxis of peritoneal cells

Results and Discussion

Stability Studies of Trichloroethylene in the Drinking Water: We are continuing investigations to establish the stability of TCE-2 in emulphor: water. Emulphor is a polyethoxylated vegetable oil that has both aqueous solubility and will hold certain organic compounds with low aqueous solubility in solution. The emulphor has made analysis of TCE-2 difficult because it causes the columns in the gas chromatograph to become gummed up. Many solvent systems have been tried in order to extract the TCE-2 from the emulphor:water solution. The one that is presently working the best is an ether extraction.

The determination of TCE-2 in the 1% emulphor drinking water solution is summarized below:

One ml of the test solution is mixed with one ml of ether in a Pierce vial. The mixture is vortexed and centrifuged at 100 × g at 4°C for 10 minutes. One μl of the supernatant (upper phase) is injected into the gas chromatograph.

The samples are assayed on a Perkin-Elmer Sigma 1 Gas chromatograph fitted with a Flame Ionization Detector (FID). Parameters of the assay are as follows:

Temperatures: Injector port = 150° C
Detector port = 250° C
Column = Programmed 2 min. @ 100
10° per minute
200° held 5 min.

Carrier gas is nitrogen
Flow rate = 30 ml per min. N_2
Combustion gas flow = 18 psi H_2 (at instrument); 28 psi air (at instrument)
Attenuation = 7
Range = 10 mg/amp
Chart Speed = 2 cm/min

Table 23 shows the values for a 5 mg/ml solution of TCE-2 in 1% emulphor. The samples were withdrawn from a mouse drinking water bottle. There is a 20% loss of TCE-2 within 5 hours from the drinking water solution, after which the amount of TCE-2 remains constant over the 3-day observation period. Preliminary studies suggest that the 1% emulphor is not sufficient to hold the TCE-2 at 5 mg/ml, but is at 2.5 mg/ml. Thus in the studies where 5 mg TCE-2 was added per ml, it appears that this is closer to 4 mg/ml. Complete analysis of the water samples is being carried out along with details of the holding power of 1% emulphor. Extraction procedures are being investigated for blood, tissues, and cell culture media.

Body Weight, Fluid, and TCE-2 Consumption: Water bottles containing test solutions and mice receiving TCE-2 in their drinking water for 120 days were weighed twice weekly along with their water bottles. From this data, body weight gain, fluid consumption, and consumption of TCE-2 in mg/kg were obtained. In both the males and females, there were no TCE-2 or emulphor-related deaths. There was a minimal aggressiveness by the males.

Figures 8 through 11, computer printout graphs, show the growth curves for male and female mice receiving distilled water (N), 1% emulphor (V), 0.1 mg/ml TCE-2 (1), 1 mg/ml TCE-2 (2), 2.5 mg/ml TCE-2 (3), and 5 mg/ml TCE-2 (4). The graphs for each sex represent three cages of animals started three days apart. There were no TCE-2 or emulphor-related effects on growth during the 120 days.

Each data set was analyzed separately for dose-dependent differential changes in weight gain. Initial weights among the treatment groups differed significantly in all experiments except in the February 13 female study. A multivariate analysis was performed comparing the treatment groups at the end of each of the first 8 weeks while adjusting for the covariate, initial weight. Results of the February 9 male and February 13 female studies indicate no significant dose effect on the weight gain pattern. The dose by initial weight interaction in the February 13 male set was significant ($p < .001$), but examination of fitted weight values at later days presuming

Table 23

Stability of TCE-2 in 1% Emulphor

Time of Sample	Readings	Replicate Extractions 1	2	3	4	5	Mean
0	1	3.6841	3.6596	3.4928	3.4966	3.5326	3.5731
	2	3.6846	3.6643	3.6504	3.6265	3.9723	3.5964
5 hr	1	2.9894	2.8816	2.9796	2.6926	2.8462	2.8778
	2	2.9676	2.7225	3.0398	2.8822	2.8768	2.8978
24 hr	1	3.2552	3.0793	2.6340	2.8560	2.8716	2.9392
	2	3.2105	3.0113	2.1675	2.9681	-	-
48 hr	1	2.6162	2.6106	2.6784	2.5897	2.5989	2.6188
	2	2.7201	2.6921	2.6819	2.9702	-	-
72 hr	1	2.7324	2.6956	2.6916	2.7660	2.7542	2.7279
	2	2.8249	2.7705	2.7726	2.7387	2.8323	2.7878

A 5 mg/ml TCE-2 solution was prepared in 1% emulphor and placed in a mouse cage using a stainless steel pipper tube and fitted with a cork. At the indicated times, 5 ml was removed and 1 ml added to 1 ml of ether in a Pierce Vial. Fine replicate extractions were performed and 2 assays were performed on each replicate.

The numbers represent the area under the TCE-2 curve.

common initial weights indicate only minor differences in treatment group means. For the February 9 females the interaction was also significant ($p < .05$), while the fitted means for the vehicle and high-dose grous were generally on the order of two grams smaller than the other doses (Figure 11).

When the amount of TCE-2 consumed is calculated from the fluid consumed and the body weight, the data for male mice normalizes as shown in Figure 12. There is a dose-dependent consumption, but the slope of the curve is about 0.7. The mean dose in mg/kg/day for mice receiving TCE-2 in the drinking water is 21 ± 0.1 at 0.1 mg/ml, 243 ± 2 at 1 mg/ml, 439 ± 3 at 2.5 mg/ml, and 740 ± 6 at 5 mg/ml. It should be noted that in the case of 5 mg/ml, the concentration is most likely reduced to 4 mg/ml because of the holding power of 1% emulphor.

As with the males, daily fluid consumption in the females was reduced in the group receiving 5 mg/ml of TCE-2. The calculated mg/kg TCE-2 doses have values of 19.4 ± 0.2 at 0.1 mg/ml and 856 ± 7 at 5 mg/ml. As with the males, there is a linear dose-dependent intake, but the slope is 0.8. This is a reflection of the decreased intake with increasing TCE-2 concentrations.

Further analysis of this data shows that the fluid consumption per animal is fairly constant over the 4-month period. Thus calculations of fluid consumption/kg of weight would decrease. Computations on this hypothesis showed this to be true. When fluid consumption per kg of mouse is calculated for each month, the values in Table 24 are obtained for 1% emulphor in males. Since the dose in mg/kg is based on fluid consumption, the doses in mg/kg decrease over the 4-month exposure period. The calculated doses given above are thus a time-weighted average over the experimental period. The same trends shown in the males occurred in the females.

Reference Toxicology: All the variables indicated above under Reference Toxicology are currently being analyzed. Data for organ weights, hematology, urinalysis, coagulation values, and microsomal mixed functional oxidase and associated activities are not yet available. However, we do have some results from our studies of bone marrow DNA synthesis and stem cell growth.

To assess bone marrow DNA synthesis, bone marrow cells were collected from one femur and cells per femur determined. The cell count was adjusted to 5 x 10^7 cells/ml. Two hundred μl of cells and twenty μl of ^{125}I-IUdR at a concentration of 0.1 Ci/20 μl in 2.0 x 10^{-5}M fluorodeoxyuridine were added to each microtiter well. At 60, 120, and 180 minutes, 6 wells were harvested per animal, and the fentamoles of IUdR incorporated into the acid precipitable fraction

Figure 8

Body Weights of CD-1 Male Mice Receiving
Distilled Water, 1% Emulphor or TCE-2
in Drinking Water: Start Date 2/13/79

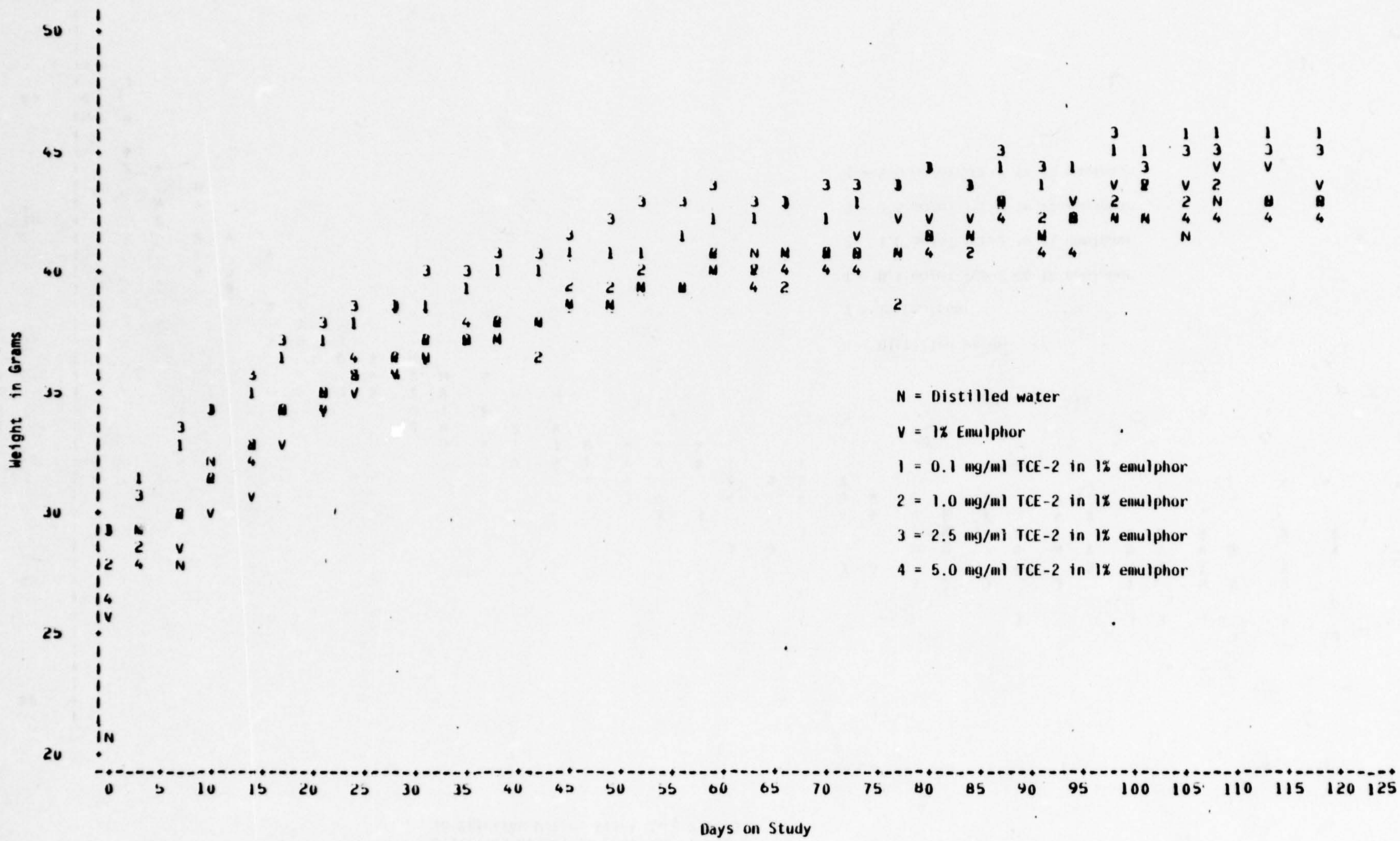


Figure 9

Body Weights of CD-1 Male Mice Receiving
Distilled Water, 1% Emulphor or TCE-2
in Drinking Water: Start Date 2/9/79

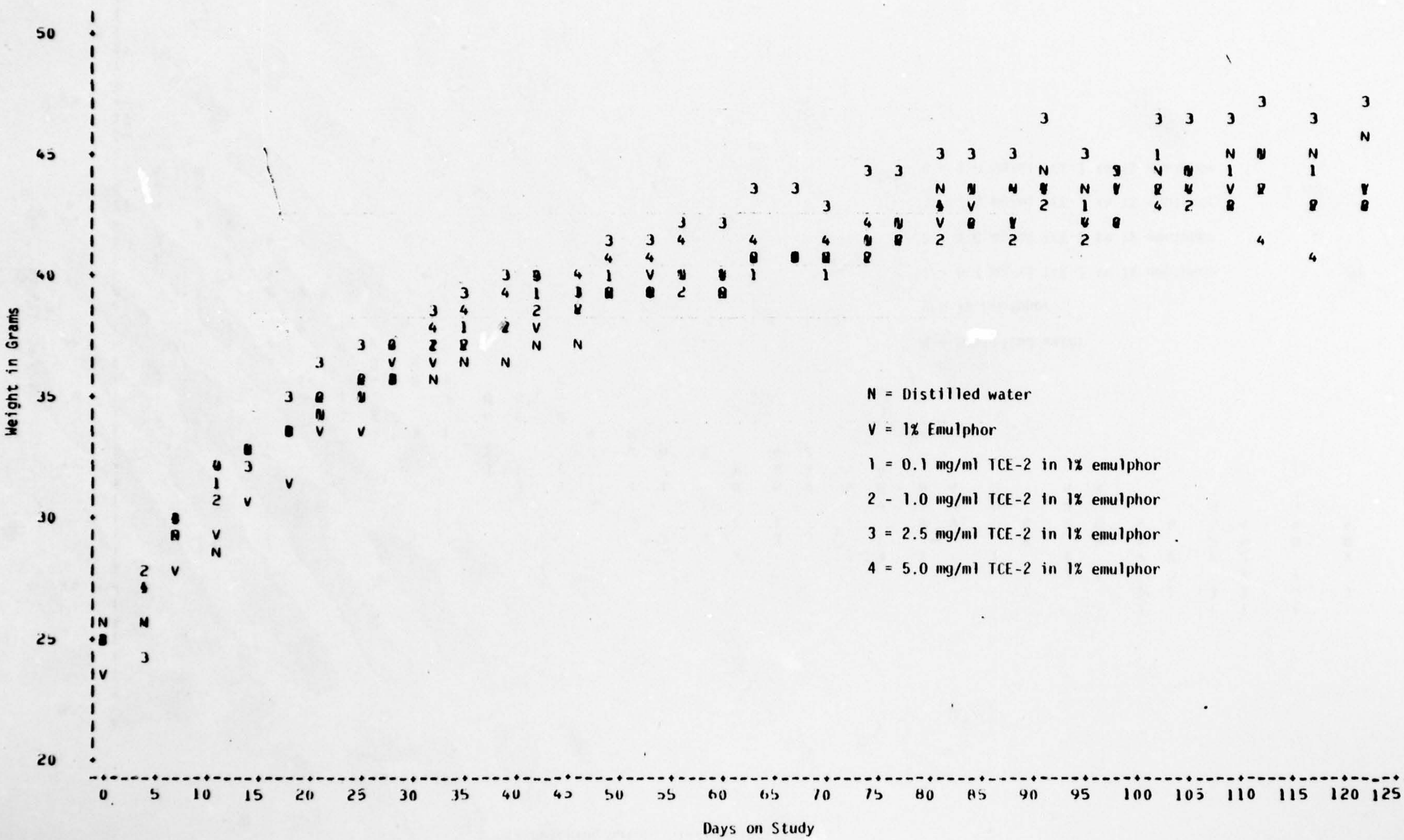


Figure 10

Body Weights of CD-1 Female Mice Receiving
Distilled Water, 1% Emulphor or TCE-2
in Drinking Water: Start Date 2/9/79

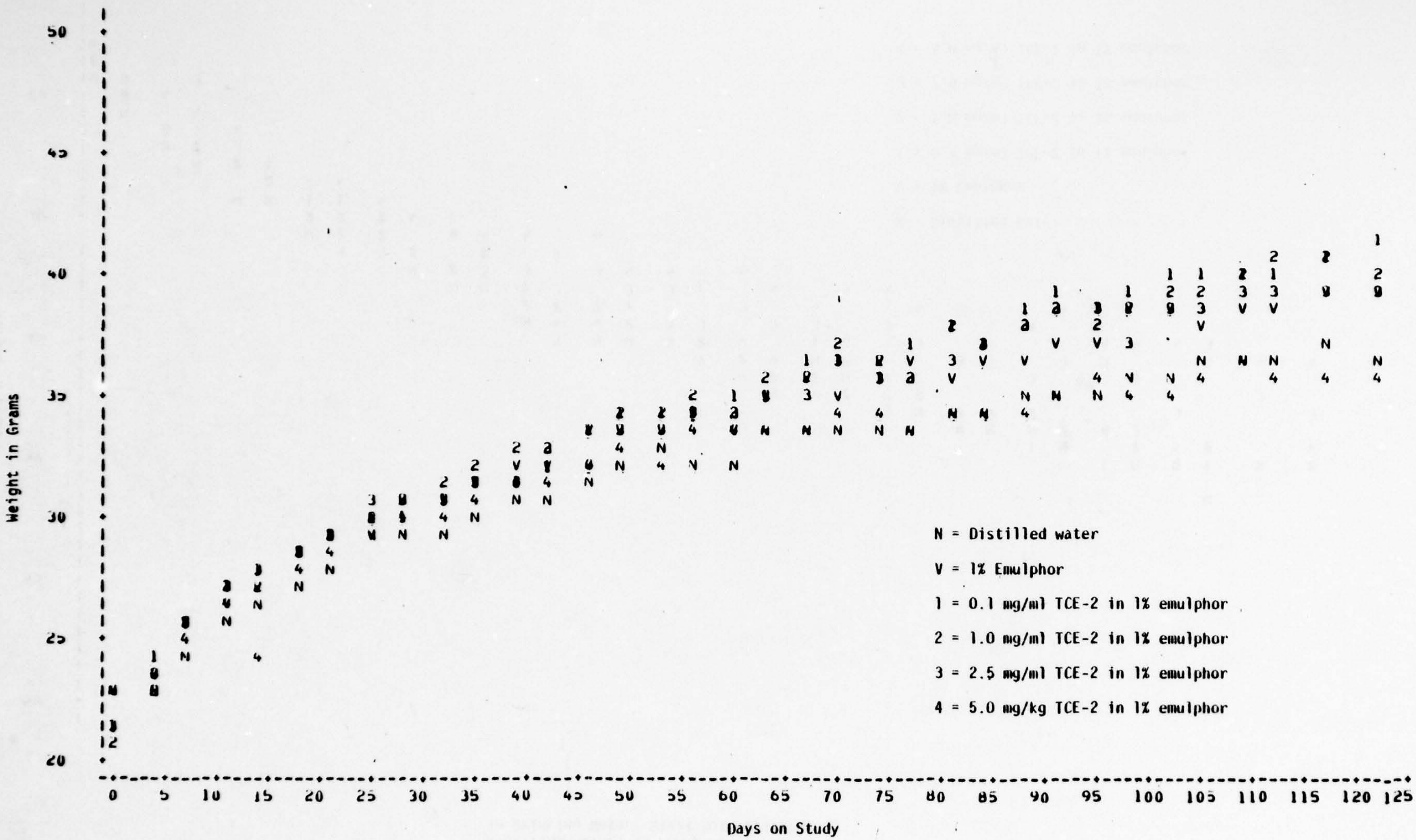
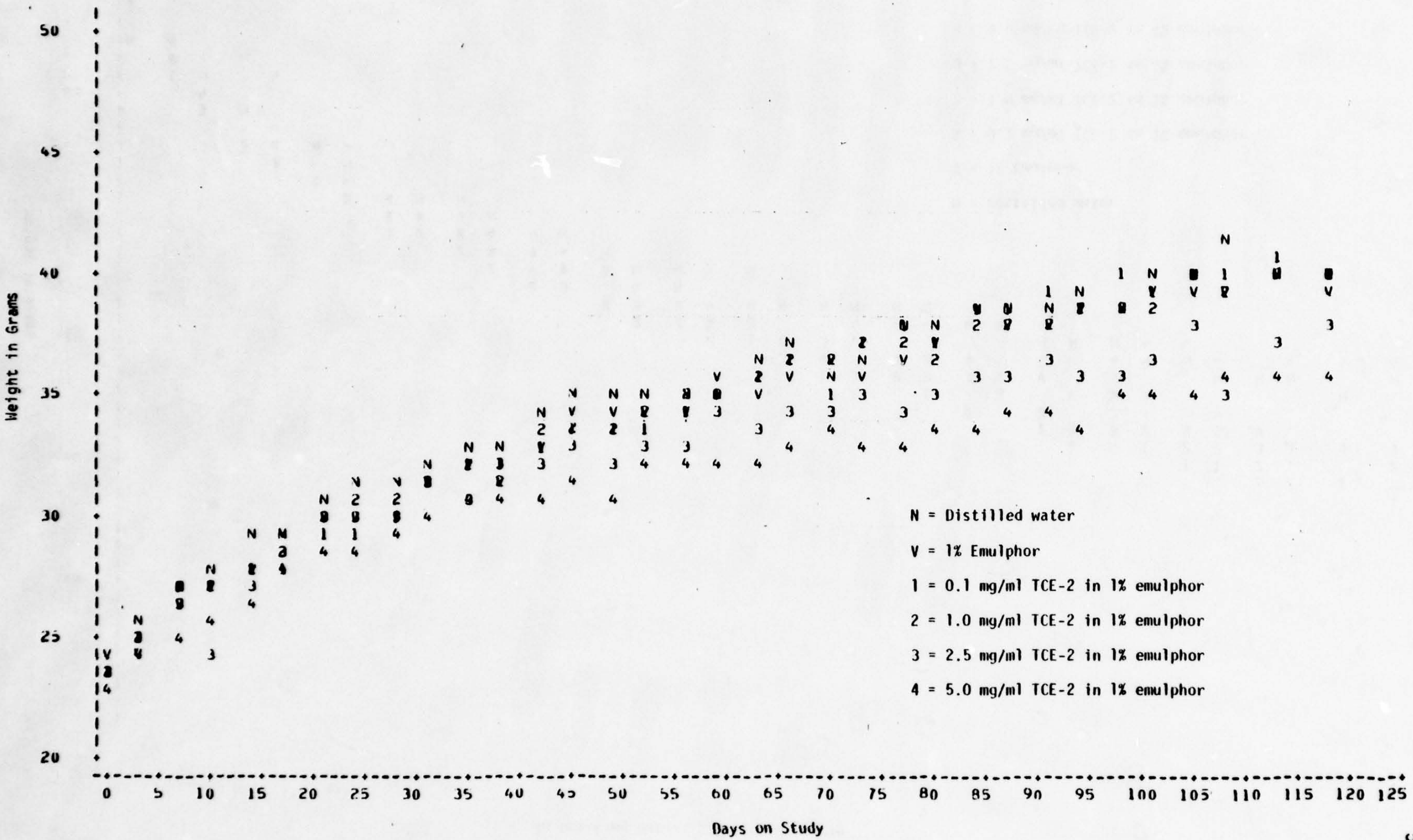


Figure 11

Body Weights of CD-1 Female Mice Receiving Distilled Water, 1% Emulphor, or TCE-2 in Drinking Water: Start Date 2/13/79

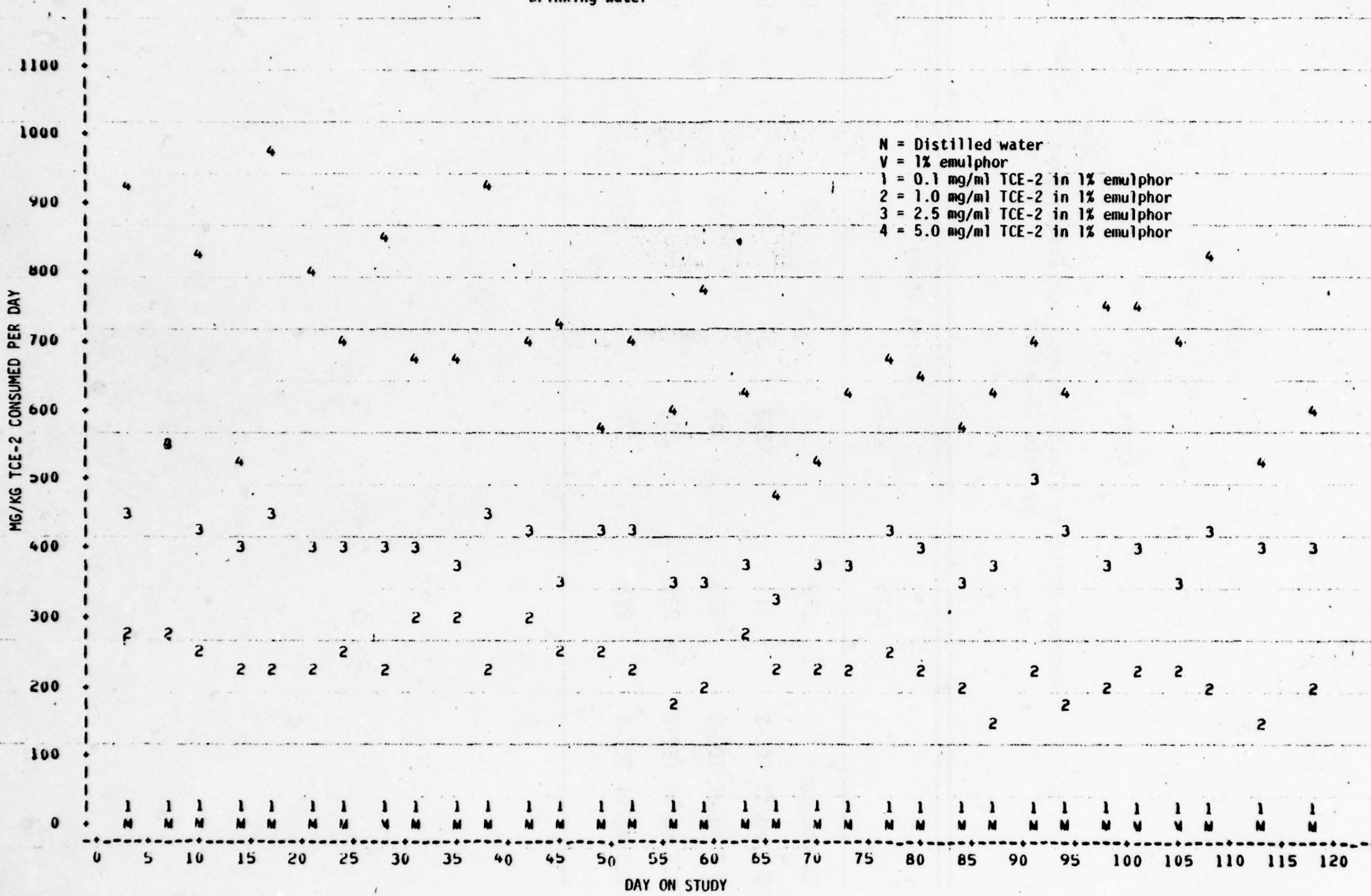


NOTE: 1 OBS HAD MISSING VALUES OR WERE OUT OF RANGE

Figure 12

Trichloroethylene Consumed (mg/kg) Per Day by CD-1 Male Mice Receiving Distilled Water, 1% Emulphor, and TCE-2 in the Drinking Water

14:43 MONDAY, JULY 2, 1979 7



NOTE: 6 OBS HAD MISSING VALUES

Table 24

Average ml/kg of Fluid Consumed per Thirty Days
by Male CD-1 Mice

	Days on Study			
	1-30	30-60	60-90	90-120
1% emulphor	258	205	185	168
0.1 mg/ml TCE-2	211	203	181	164
1.0 mg/ml TCE-2	228	209	203	185
2.5 mg/ml TCE-2	225	204	197	195
5.0 mg/ml TCE-2	225	172	162	155

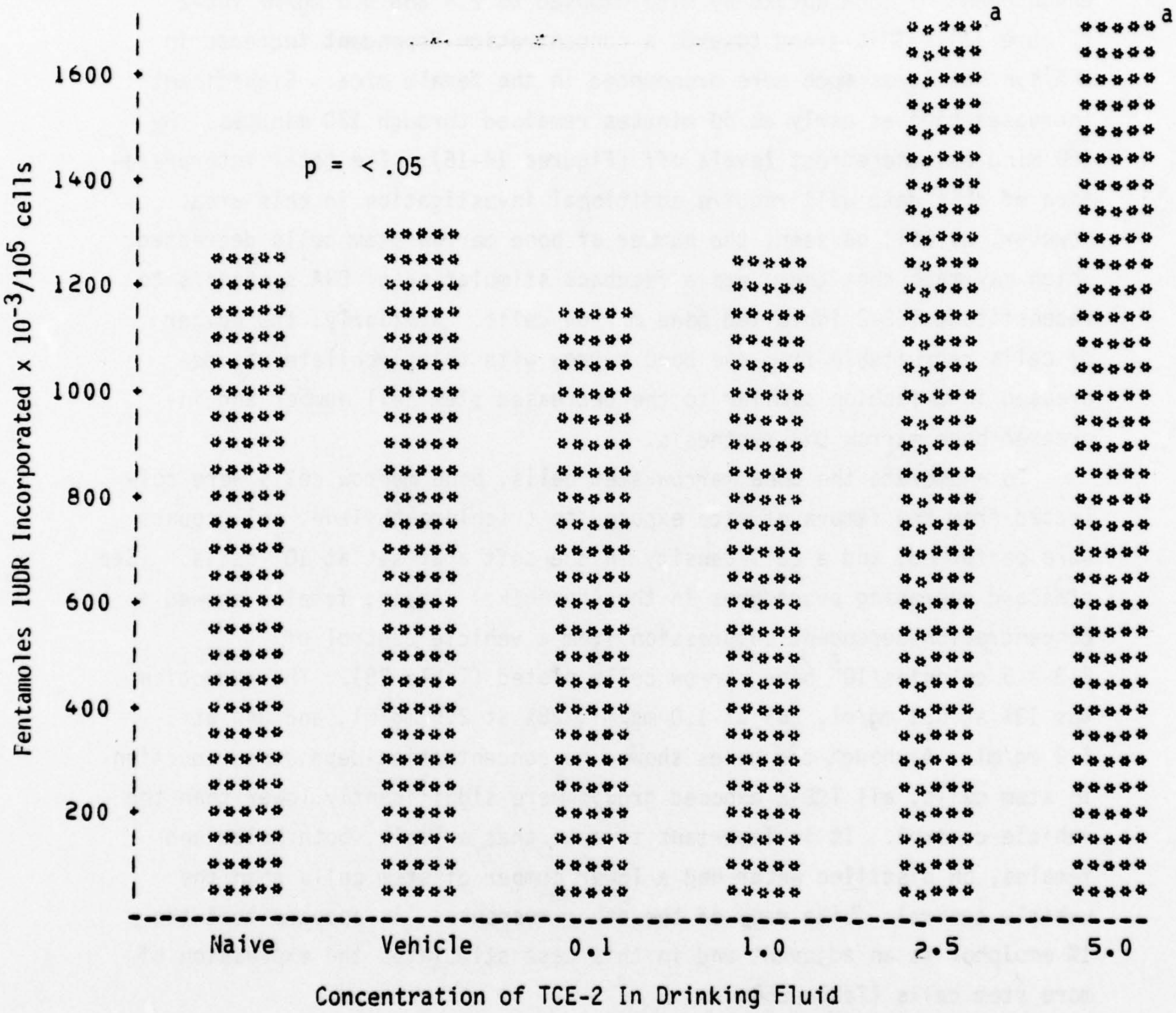
were calculated. In male mice there was a trend towards a concentration-dependent increase in IUdR uptake at 60 minutes and 120 minutes, but there was no statistical difference. At 180 minutes, there was a pronounced enhancement of IUdR uptake by mice exposed to 2.5 and 5.0 mg/ml TCE-2 (Figure 13). This trend towards a concentration-dependent increase in DNA synthesis was much more pronounced in the female mice. Significant increases seen as early as 60 minutes remained through 120 minutes. By 180 minutes, the effect levels off (Figures 14-16). The total interpretation of this data will require additional investigation in this area. However, as will be seen, the number of bone marrow stem cells decreased, which may mean that there was a feedback stimulation of DNA synthesis to reconstitute TCE-2 inhibited bone marrow cells. Similarly, the number of cells recruitable from the bone marrow with thioglycollate was decreased in a fashion similar to the decreased stem cell number and increased bone marrow DNA synthesis.

To enumerate the bone marrow stem cells, bone marrow cells were collected from the femurs of mice exposed to trichloroethylene, cell counts were performed, and a cell density in the soft agar set at 10^6 cells. (See standard operating procedures in the Appendix.) Again, females showed a concentration-dependent suppression from a vehicle control of 213 ± 5 colonies/10^5 bone marrow cells plated (Table 25). The reduction was 18% at 0.1 mg/ml, 25% at 1.0 mg/ml, 28% at 2.5 mg/ml, and 34% at 5.0 mg/ml. Although the males showed no concentration-dependent reduction in stem cells, all TCE-2 exposed groups were significantly lower than the vehicle control. It is important to note that animals, both males and females, on distilled water had a lower number of stem cells than the vehicle control. Like many of the other responses, it appears that the 1% emulphor is an adjuvant and in this case stimulates the expression of more stem cells (Table 25).

Cell Mediated Immunity: The status of cell-mediated immunity was assessed by measuring the delayed type hypersensitivity (DTH) response to sheep erythrocytes. The procedure for this assay is provided in the standard operating

Figure 13

DNA Synthesis (^{125}I IUDR Uptake) in Bone Marrow Cells of Male Mice Exposed To Trichloroethylene in Drinking Water For 120 Days: 180 Minutes Incubation

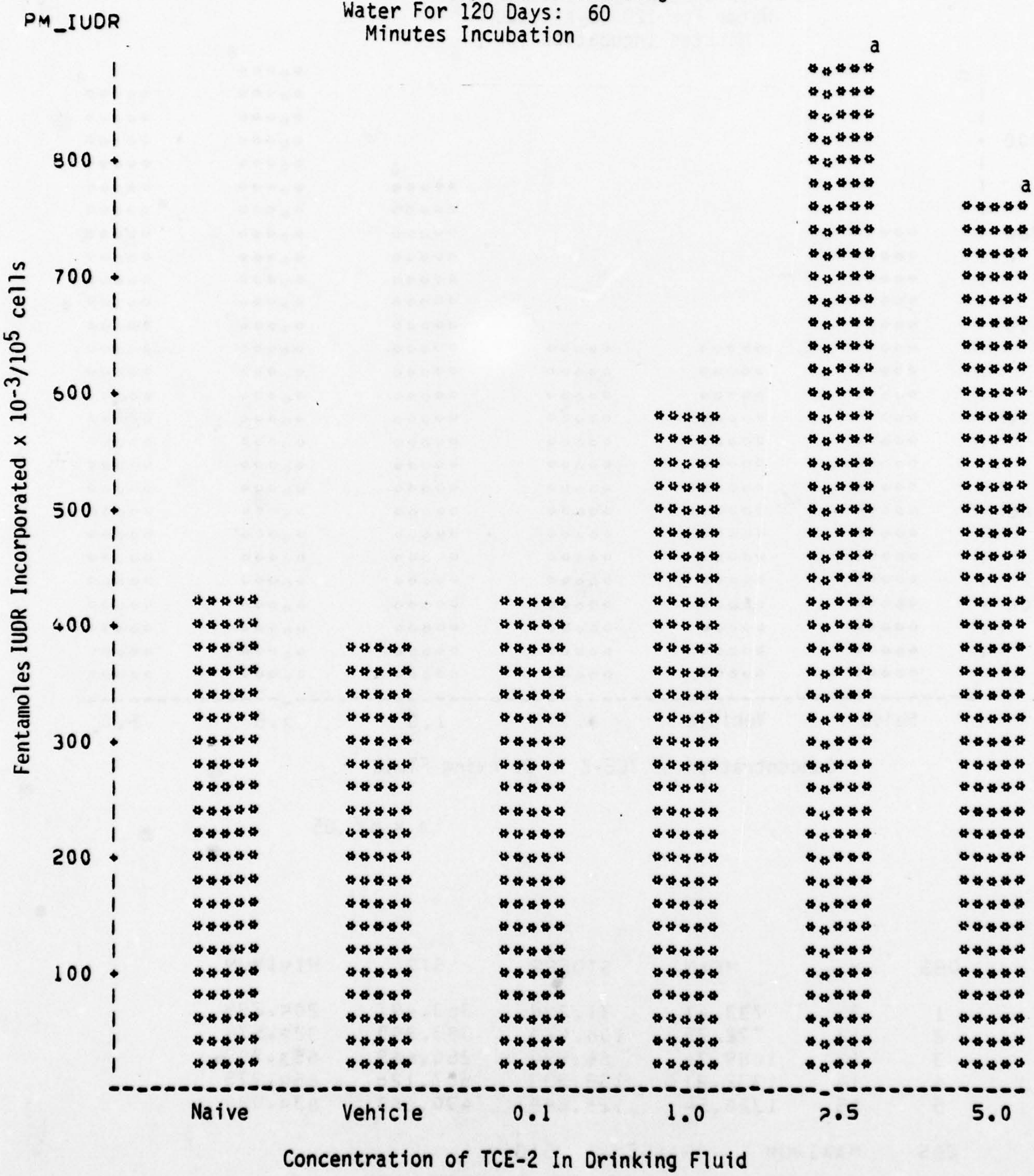


OBS	N	MEAN	STDERR	STD	MINIMUM
1	25	1278.49	86.428	432.141	530.88
2	14	1143.27	90.118	337.191	665.33
3	15	1263.41	114.039	441.670	851.43
4	14	1675.13	110.035	411.714	1122.10
5	15	1696.89	171.525	664.313	767.32

OBS	MAXIMUM	RANGE	SIG
1	2405.77	1874.89	NOT SIGNIF AT .05 LEVEL
2	1707.25	1041.92	NOT SIGNIF AT .05 LEVEL
3	2233.05	1381.62	NOT SIGNIF AT .05 LEVEL
4	2454.41	1332.31	SIGNIF AT .05 LEVEL
5	2978.20	2210.87	SIGNIF AT .05 LEVEL

Figure 14

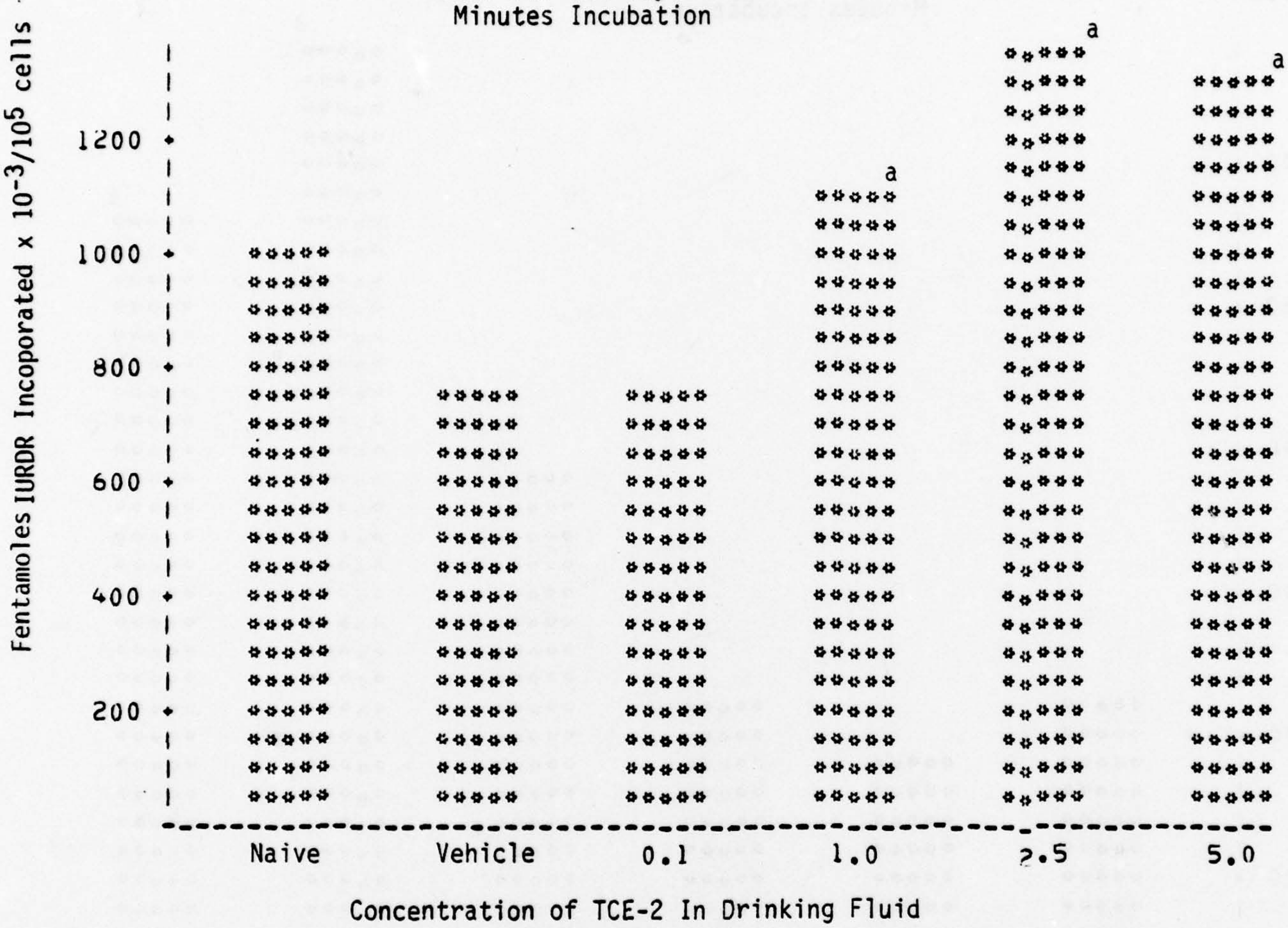
DNA Synthesis (^{125}I IUDR Uptake) in Bone Marrow Cells of Female Mice Exposed To Trichloroethylene in Drinking Water For 120 Days: 60 Minutes Incubation



a = p < .05

Figure 15

DNA Synthesis (^{125}I IUDR Uptake) in Bone Marrow Cells of Female Mice Exposed To Trichloroethylene In Drinking Water For 120 Days: 120 Minutes Incubation



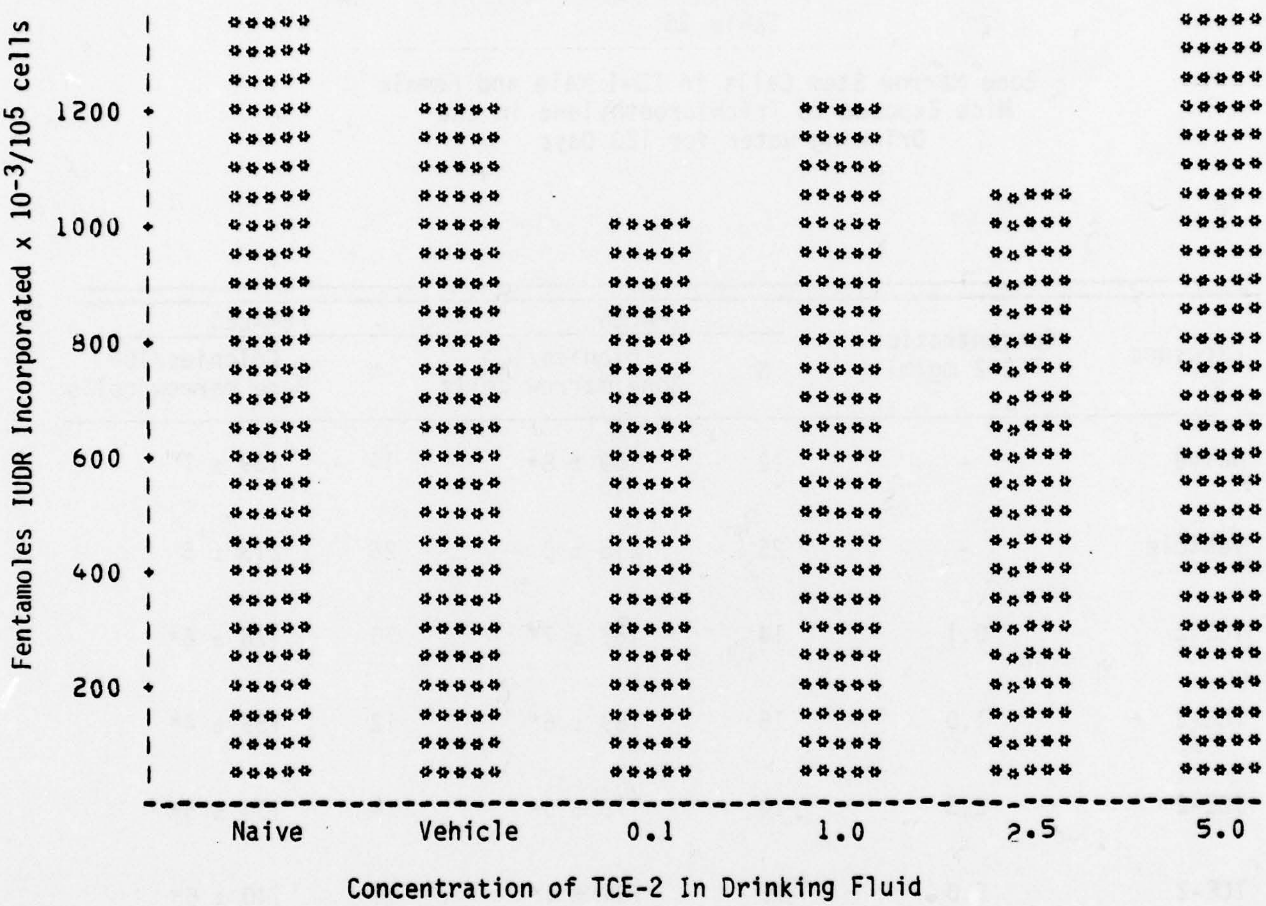
a = p< .05

OBS	N	MEAN	STDERR	STD	MINIMUM
1	26	733.41	71.288	363.497	205.299
2	13	772.75	106.473	383.893	322.574
3	14	1089.74	66.994	250.668	653.708
4	14	1332.81	135.535	507.126	659.975
5	15	1320.54	126.688	490.660	638.084

OBS	MAXIMUM	RANGE	SIG
1	1807.46	1602.16	NOT SIGNIF AT .05 LEVEL
2	1563.11	1240.54	NOT SIGNIF AT .05 LEVEL
3	1637.22	983.51	SIGNIF AT .05 LEVEL
4	2201.89	1541.91	SIGNIF AT .05 LEVEL
5	2376.86	1738.78	SIGNIF AT .05 LEVEL

Figure 16

DNA Synthesis (^{125}I IUDR Uptake) in Bone Marrow Cells of Female Mice Exposed to Trichloroethylene in Drinking Water For 120 Days: 180 Minutes Incubation



OBS	N	MEAN	STDERR	STD	MINIMUM
1	26	1224.02	71.345	363.791	812.997
2	13	1015.12	92.629	333.980	391.513
3	14	1211.23	89.828	336.107	685.284
4	14	1035.71	104.448	390.810	464.262
5	15	1326.92	70.017	271.175	908.478

OBS	MAXIMUM	RANGE	SIG
1	2376.30	1563.30	NOT SIGNIF AT .05 LEVEL
2	1618.11	1226.60	NOT SIGNIF AT .05 LEVEL
3	1800.82	1115.54	NOT SIGNIF AT .05 LEVEL
4	1917.30	1453.03	NOT SIGNIF AT .05 LEVEL
5	1835.06	926.58	NOT SIGNIF AT .05 LEVEL

Table 25

Bone Marrow Stem Cells in CD-1 Male and Female Mice Exposed to Trichloroethylene in the Drinking Water for 120 Days

Exposure	Concentration TCE-2 mg/ml	MALES N	MALES Colonies/10^5 Bone marrow cells	FEMALES N	FEMALES Colonies/10^5 Bone marrow cells
Naive	-	12	159 ± 8*	14	189 ± 7
Vehicle	-	25	215 ± 3	26	213 ± 5
TCE-2	0.1	14	183 ± 7*	13	176 ± 5*
TCE-2	1.0	15	189 ± 6*	12	159 ± 4*
TCE-2	2.5	14	178 ± 5*	14	154 ± 5*
TCE-2	5.0	15	184 ± 4*	15	140 ± 6*

CD-1 male and female mice were exposed to distilled water, 1% emulphor (vehicle) or TCE-2 at the indicated concentrations in their drinking water. At the end of 120 days, bone marrow cells were collected from a femur and the number of stem cells determined by growth in soft agar. Numbers represent mean ± standard error derived from the number of mice indicated in column N. * = $P < .05$, as compared to vehicle treated group.

procedures (see Appendix). The DTH response was measured four days after the sensitization. Female mice showed suppressed DTH response at all concentration levels (Table 26). The suppression was not concentration-related, as seen in a 45%, 29.4%, 51.5%, and 47.2% inhibition at 0.1, 1.0, 2.5, and 5.0 mg/ml TCE-2 in the drinking water. Since this is not a dose-dependent response, it may be that TCE-2 exposure is affecting a helper cell, an amplifier cell, and/or a cell type responsible for expression of the DTH response. The males showed no changes in DTH response (Table 27). These data suggest that TCE-2 has an adverse effect on cell mediated immunity. The specific cell type response is not known, but could be either a T lymphocyte or the macrophage. We will be looking for *in vitro* relations to this response.

Spleen cell responsiveness to the mitogens was assessed by exposing mice to 1% emulphor (vehicle), distilled water, or TCE-2. The mice were anesthetized with chloroform and blood collected by cardiac puncture. The spleens were asceptically removed, weighed, and a single cell suspension prepared by pushing the spleen through a 100-mesh stainless steel screen. Cell counts were performed and cell density adjusted to 5 x 10^6 cells per ml in RPMI 1640 medium with 15% calf serum (see standard operating procedures, Appendix, for details of the method). Three concentrations of Conconavalin A and lipopolysaccharide (1, 5, and 10 μg/well) are added to the microtiter well along with 5 x 10^6 spleen cells. Mitogenic response is allowed to develop over a 48-hour incubation period (10% CO_2, 95% O_2, 95% relative humidity) followed by an 18-hour pulse of ^{125}I-IUdR. The cultures are radioassayed and the mitogenic response calculated as a relative response = cpm/culture with mitogens = cpm/culture without mitogens. In this experiment, the females responded better than the males and the response was much better with Conconavalin A than bacterial lipopolysaccharide. Although the data is still being analyzed, it appears that there are alterations in mitogen response to Conconavalin A in female mice.

Humoral Immunity: Spleen antibody forming cells (AFC) to sheep erythrocytes require the cooperation of macrophages, T lymphocytes, and B lymphocytes. This response assesses the staus in part of humoral immunity. The assay method followed is provided in the standard operating procedure in the Appendix. AFC response was measured on days 4 and 5 after sensitization with sheep erythrocytes and direct IgM forming cells were quantified. Tables 28 and 29 show the results of this study.

Table 26

Delayed Type Hypersensitivity Response to
Sheep Erythrocytes in CD-1 Female Mice
Exposed to Trichloroethylene in the Drinking Water
For 120 Days

Exposure	N	TCE-2		Stimulation Index	Percent Inhibition
		Concentration (mg/ml)	Dose (mg/kg/day)		
Vehicle	20	-	-	5.41 ± 0.43	-
Distilled water	15	-	-	4.54 ± 0.59	-
TCE-2	15	0.1 mg/ml	19 ±.1	3.00 ± 0.50 *	45%
TCE-2	15	1.0 mg/ml	211 ± 1	3.82 ± 0.60 *	29.4%
TCE-2	15	2.5 mg/ml	484 ± 4	2.60 ± 0.32 *	51.1%
TCE-2	15	5.0 mg/ml	856 ± 11	2.86 ± 0.20 *	47.2%

Groups of CD-1 female mice were exposed to trichloroethylene in drinking water for 120 days. Delayed type hypersensitivity to sheep erythrocytes was measured 4 days after sensitization. Vehicle = 1% emulphor in distilled water. Numbers represent the mean ± standard error. N = number per group. *P < .05 as compared to vehicle treated group.

Table 27

Delayed Type Hypersensitivity Response to Sheep Erythrocytes in CD-1 Male Mice Exposed to Trichloroethylene in the Drinking Water for 120 Days

Exposure	N	Concentration TCE-2 (mg/ml)	Dose Consumed (mg/kg/day)	Stimulation Index	Percent Inhibition
Vehicle	19	-	-	4.04 ± 0.40	-
Distilled water	15	-	-	2.85 ± 0.36	28.5%
TCE-2	15	0.1 mg/ml	20 ± 0.1	2.99 ± 0.34	26.0%
TCE-2	15	1.0 mg/ml	229 ± 2	3.16 ± 0.30	-
TCE-2	14	2.5 mg/ml	409 ± 3	4.83 ± 0.70	-
TCE-2	15	5.0 mg/ml	693 ± 6	4.13 ± 0.58	-

Groups of CD-1 male mice were exposed to trichloroethylene in drinking water for 120 days. Delayed type hypersensitivity to sheep erythrocytes was measured 4 days after sensitization. Vehicle = 1% emulphor in distilled water. Concentration of TCE-2 mg/ml added to the drinking water (1% emulphor in distilled water). Dose consumed in mg/kg/day calculated from the concentration and volume consumed. N = number of mice per group. Data shown as mean ± standard error.

Table 28

Spleen Antibody Forming Cells (AFC) in CD-1 Male Mice Exposed to Trichloroethylene (TCE-2) in the Drinking Water for 120 Days

Exposure	No.	Concentration TCE-2 in Drinking Water	Day 4		Day 5	
			AFC/spleen $\times 10^5$	AFC/10^6 cells	AFC/spleen $\times 10^5$	AFC/10^6 cells
Vehicle	7	-	2.53 ± 0.24	1832 ± 306	1.16 ± 0.11	773 ± 161
Naive	7	-	2.66 ± 0.27	1534 ± 159	1.11 ± 0.11	577 ± 99
TCE-2	7	0.1 mg/ml	1.74 ± 0.28	791 ± 183*	1.39 ± 0.31	508 ± 93
TCE-2	7	1.0 mg/ml	2.30 ± 0.23	2063 ± 224	1.16 ± 0.15	637 ± 96
TCE-2	7	2.5 mg/ml	2.37 ± 0.22	1622 ± 274	0.92 ± 0.18	671 ± 143
TCE-2	7	5.0 mg/ml	2.56 ± 0.32	1921 ± 312	0.96 ± 0.07	599 ± 65

Groups of CD-1 male mice were exposed to trichloroethylene in drinking water for 120 days. Mice were immunized with sheep erythrocytes 4 or 5 days prior to assay. Spleen antibody forming cells (plaque forming cells) were enumerated and expressed as ACF per total spleen and AFC per 10^6 spleen cells. Numbers represent mean ± standard error derived from the number in the group. N = number in group. Vehicle = 1% emulphor. *$P < 0.05$ as compared to vehicle control.

Table 29

Spleen Antibody Forming Cells (AFC) in CD-1 Female Mice Exposed to Trichloroethylene (TCE-2) in the Drinking Water for 120 Days

Exposure	No.	Concentration TCE-2 in Drinking Water	Day 4		Day 5	
			AFC/spleen x 10^5	AFC/10^6 cells	AFC/spleen x 10^5	AFC/10^6 cells
Vehicle	7	-	1.93 ± 0.08	2038 ± 154	0.55 ± 0.08	272 ± 67
Naive	7	-	2.27 ± 0.35	1426 ± 173	0.46 ± 0.09	206 ± 54
TCE-2	7	0.1 mg/ml	1.36 ± 0.07*	1718 ± 231	0.50 ± 0.04	313 ± 36
TCE-2	7	1.0 mg/ml	1.97 ± 0.18	1289 ± 335	0.57 ± 0.05	323 ± 56
TCE-2	7	2.5 mg/ml	1.23 ± 0.15*	639 ± 100*	0.51 ± 0.05	257 ± 46
TCE-2	7	5.0 mg/ml	1.36 ± 0.21*	732 ± 129*	0.41 ± 0.04	246 ± 17

Groups of CD-1 female mice were exposed to trichloroethylene in drinking water for 120 days. Mice were immunized with sheep erythrocytes 4 or 5 days prior to assay. Spleen antibody forming cells (plaque forming cells) were enumerated and expressed as AFC per total spleen and AFC/10 spleen cells. Numbers represent mean ± standard error derived from number in the group. N = number in group. Vehicle = 1% emulphor. *$P < 0.05$ as compared to vehicle control.

Excellent antibody response was generated in both males and females as seen in AFC/10^6 spleen cells of 1832 and 2038 in peak day (4) for males and females, respectively. Male mice (Table 28) exposed to 0.1 mg/ml of TCE-2 showed a reduction of 57% as compared to vehicle control. The reason for this effect, which occurred at the lowest dose and was the only significant ($p < .05$) effect seen, is that the spleens of this group were larger than normal. The mean spleen weight expressed as mg percent of body weight was 387 compared to 282 mg for the vehicle control and 0.97% of body weight at compared to 0.62% for the vehicle control group. These males had shown signs of biting which most likely was responsible for the results. Table 30 details the data derived from the male mice. The female mice showed a 30%, 37%, and 31% inhibition of AFC/spleen at TCE-2 concentrations of 0.1, 2.5, and 5.0 mg/ml. As with the DTH response, there was no apparent concentration dependency. Calculated as specific activity, i.e. AFC/10^6 spleen cells, mice exposed to 2.5 mg/ml and 5.0 mg/ml showed suppressions of 69% and 64%, respectively. The data for day 5 shows that this inhibition is not a simple delay because there was no rebound increase over vehicle control. Again, day 4 was the peak day of response. Table 31 details the data derived from the female mice. Additional analysis is available upon request.

The data on spleen antibody forming cells is congruent with the serum hemagglutination titer to sheep erythrocytes. Male mice showed no significant reduction in antibody titers to sheep erythrocytes (Table 32). The titers are expressed as LOG_2 of the reciprocal of the titer. Serum for 25 vehicle-treated mice gave a titer of 8.842 ± 0.131 S.E. This reconverts back to a titer of 1/458. The highest titer in males was in the mice on distilled water with a titer of 9.180, converting to a titer of 1/579. Female mice on distilled water also had a higher titer, 10.606, than the vehicle-exposed animals, 9.962 (Table 33). These convert to titers of 1/1559 and 1/997, respectively. There was a concentration-dependent suppression of antibody response in the female mice. Transforming the data back to titers, the inhibitions at 1, 2.5, and 5 mg/ml were 30, 45, and 64%, respectively. The two sets of data, that is, spleen antibody forming cells and serum antibody levels from different groups of mice, support each other and suggest that TCE-2 suppresses humoral immune response. The complexity of the cell types involved in this response make it difficult to suggest a cell target for TCE-2. The *in vitro* studies may add information which may help in explaining the effect and provide a prediction of toxicity.

TABLE 30

SUMMARY TABULATION: ANTIBODY FORMING CELLS DAY 4

SEX=M VEHICLE

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	46.728571	6.952629	39.10000	58.60000
SPLEENWT	7	282.857143	77.921817	195.00000	404.00000
SPLEENPC	7	0.619243	0.213569	0.41126	1.03325
CELLSMG	7	5.379364	1.034768	4.30078	7.10788
PLA_SPL	7	253220.428571	64281.865361	167849.00000	349799.00000
PLA_106	7	1832.728311	811.538951	714.53284	2956.73504

---------------------------- SEX=M 0.1 MG/ML ----------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	40.028571	4.153599	35.100000	45.90000
SPLEENWT	7	387.142857	120.786904	220.000000	549.00000
SPLEENPC	7	0.972474	0.291533	0.504587	1.35969
CELLSMG	7	6.288559	0.603620	5.474801	6.88636
PLA_SPL	7	174256.142857	75277.904176	58899.000000	260699.00000
PLA_106	7	790.864014	484.533324	194.497103	1382.83498

---------------------------- SEX=M 1.0 MG/KG ----------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	43.157143	3.851345	39.00000	49.80000
SPLEENWT	7	216.714286	51.622716	144.00000	314.00000
SPLEENPC	7	0.506052	0.138197	0.35910	0.78697
CELLSMG	7	5.256166	0.986850	4.43534	6.91866
PLA_SPL	7	229841.857143	59585.424872	137099.00000	333299.00000
PLA_106	7	2063.543341	593.388351	1380.26909	3234.63725

---------------------------- SEX=M 2.5 MG/KG ----------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	44.257143	2.800510	41.20000	47.10000
SPLEENWT	7	268.142857	24.721304	230.00000	303.00000
SPLEENPC	7	0.607519	0.063184	0.51225	0.70531
CELLSMG	7	5.939395	1.884332	3.93493	8.72609
PLA_SPL	7	237384.714286	59090.028165	140999.00000	324599.00000
PLA_106	7	1622.127519	727.661652	837.36420	2618.95261

---------------------------- SEX=M 5.0 MG/KG ----------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	43.942857	2.558552	41.70000	49.20000
SPLEENWT	7	218.714286	45.922813	166.00000	302.00000
SPLEENPC	7	0.500204	0.116078	0.37900	0.71226
CELLSMG	7	6.411707	1.030816	5.14865	8.22727
PLA_SPL	7	255913.285714	85902.802216	155099.00000	372899.00000
PLA_106	7	1920.661370	826.945323	841.08094	3021.04586

---------------------------- SEX=M Naive ----------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	46.757143	5.233182	41.00000	54.10000
SPLEENWT	7	222.000000	22.590558	178.00000	243.00000
SPLEENPC	7	0.478574	0.064142	0.39926	0.59268
CELLSMG	7	7.758597	1.353442	6.20870	10.43258
PLA_SPL	7	266170.428571	73110.298278	157199.00000	340799.00000
PLA_106	7	1534.125081	420.438146	867.91007	1956.48628

TABLE 31
SUMMARY TABULATION: ANTIBODY FORMING CELLS
DAY 4

VEHICLE

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	41.800000	6.038212	36.30000	51.10000
SPLEENWT	7	244.571429	37.638000	204.00000	321.00000
SPLEENPC	7	0.598735	0.147340	0.45102	0.88430
CELLSMG	7	3.913113	0.357185	3.29487	4.44118
PLA_SPL	7	193113.285714	20027.267127	169499.00000	227099.00000
PLA_106	7	2037.864552	407.253967	1394.22222	2542.86252

------------------------- SEX=F 0.1 MG/ML-------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	36.757143	3.599471	33.80000	41.90000
SPLEENWT	7	257.142857	56.386844	203.00000	347.00000
SPLEENPC	7	0.693162	0.083668	0.60059	0.82816
CELLSMG	7	3.316248	0.684372	1.88000	4.05231
PLA_SPL	7	136227.571429	17958.258480	110599.00000	164599.00000
PLA_106	7	1718.157517	612.183269	949.52847	2581.63357

------------------------- SEX=F 1.0MG/ML-------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	38.528571	3.202454	33.00000	43.00000
SPLEENWT	7	250.571429	34.707622	194.00000	300.00000
SPLEENPC	7	0.650718	0.078960	0.56522	0.78534
CELLSMG	7	6.827176	1.236680	4.11340	7.52000
PLA_SPL	7	196570.428571	49023.420059	139199.00000	258599.00000
PLA_106	7	1288.657260	888.063859	674.44238	3207.58897

------------------------- SEX=F 2.5MG/ML-------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	41.714286	2.939631	37.300000	45.10000
SPLEENWT	7	211.000000	32.710854	173.000000	251.00000
SPLEENPC	7	0.511079	0.105740	0.385809	0.67292
CELLSMG	7	9.008127	0.733326	8.091633	10.03448
PLA_SPL	7	123170.428571	39216.055993	72399.000000	175799.00000
PLA_106	7	639.290677	263.843391	284.957839	1027.41412

------------------------- SEX=F 5.0MG/ML-------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	39.442857	3.884524	31.500000	43.00000
SPLEENWT	6	251.166667	44.512545	185.000000	297.00000
SPLEENPC	6	0.655348	0.151794	0.444712	0.88571
CELLSMG	6	7.518127	1.148591	6.012821	8.91892
PLA_SPL	6	136232.333333	49170.668764	45999.000000	188399.00000
PLA_106	6	732.865685	316.955529	171.986631	1033.20826

------------------------- SEX=F NAIVE -------------------------

VARIABLE	N	MEAN	STANDARD DEVIATION	MINIMUM VALUE	MAXIMUM VALUE
MOUSEWT	7	42.428571	6.716575	33.80000	55.60000
SPLEENWT	7	220.000000	17.511901	189.00000	240.00000
SPLEENPC	7	0.525069	0.056982	0.43165	0.58505
CELLSMG	7	7.145461	1.912986	4.07500	9.36279
PLA_SPL	7	227384.714286	94042.816996	117799.00000	398399.00000
PLA_106	7	1426.152397	459.340515	862.67312	2074.93122

Table 32

Hemagglutination Titers in CD-1 Male Mice
Exposed to Trichloroethylene (TCE-2) in the Drinking Water for 120 Days

Exposure	No.	Concentration TCE-2 in Drinking Water	LOG_2 Hemagglutination Titer	Activity Titer
Vehicle	25	-	8.842 ± 0.131	1/458
Distilled water	14	-	9.180 ± 0.294	1/579
TCE-2	15	0.1 mg/ml	8.589 ± 0.118	1/384
TCE-2	15	1.0 mg/ml	8.722 ± 0.190	1/422
TCE-2	15	2.5 mg/ml	8.679 ± 0.133	1/410
TCE-2	15	5.0 mg/ml	9.122 ± 0.107	1/557

Groups of CD-1 male mice were exposed to trichloroethylene in drinking water for 120 days. Hemagglutination titers to sheep erythrocytes were determined from serum separated from blood collected by cardiac puncture. The mice were immunized against sheep erythrocytes six days prior to assay. Numbers represent the mean ± standard error derived from the number in the group. N = number of mice per group. Vehicle = 1% emulphor. Titer expressed as the LOG_2 of the reciprocal of the first dilution not to show agglutination. There were no significant differences among the groups.

Table 33

Hemagglutination Titers in CD-1 Female Mice
Exposed to Trichloroethylene in the Drinking Water for 120 Days

Exposure	No.	Concentration TCE-2 in Drinking Water	LOG_2 Hemagglutination Titers	Antibody of Titers	% Inh.
Vehicle	25	-	9.962 ± 0.251	1/997	
Distilled water	14	-	10.608 ± 0.286	1/1559	
TCE-2	15	0.1 mg/ml	10.255 ± 0.300	1/1197	
TCE-2	15	1.0 mg/ml	9.455 ± 0.236	1/701	30%
TCE-2	14	2.5 mg/ml	9.108 ± 0.214*	1/551	45%
TCE-2	15	5.0 mg/ml	8.589 ± 0.182*	1/385	64%

Groups of CD-1 female mice were exposed to trichloroethylene in drinking water for 120 days. Hemagglutination titers to sheep erythrocytes were determined from serum separated from cardiac blood. The mice were immunized against sheep erythrocytes six days prior to assay. Numbers represent the mean ± standard error derived from the number in the group. N = number of mice per group. Vehicle + 1% emulphor. Titers are expressed as LOG_2 of the reciprocal of the dilution not to show agglutination.
* $P < 0.05$ as compared to vehicle group.

Functional Activity of the Reticuloendothelial System: Vascular clearance rate and organ uptake of ^{51}Cr sheep erythrocytes was evaluated in mice exposed to TCE-2 for 120 days. This data is presently being analyzed.

The recruitability of peritoneal exudate cells in these mice was also studied. The ability of the cells to respond to a stimulus in the peritoneal cavity and migrate there is a complex function, whose rate limiting step is believed to be the status of the bone marrow. The mice were assessed for their ability to recruit cells into the peritoneal cavity in response to thioglycollate. Not only were the number of cells determined, but the numbers of cells that adhered, their ability to move towards the chemotactic factor, and their ability to phagocytize opsonized sheep erythrocytes. The effects of various doses of TCE-2 on recruitability of cells into the peritoneal cavity of the mouse is presented in Table 34. The data obtained from female mice were not significantly different from each other at the $p < .05$ level using the Duncan's Multiple Range Test. In males, a complex response was observed. Vehicle treatment was not found to be significantly different from the naive-treated animal, while all four TCE-2 exposed groups were significantly depressed when compared to the vehicle controls. However, they were not found to be different from each other, that is, there was no dose response. This assay is a crude indicator of the ability of cells to migrate in response to a stimulus. This is one situation where the female mice were not altered but the males were. As indicated earlier, the number of bone marrow stem cells in female mice was decreased more dramatically than were the males. It seems that some relationship between these two assays should eventually show up. We believe that the recruitability in the peritoneal cavity is an important assay and that we should work towards refining it and discussing it with our colleagues as to the overall implications.

The ability of peritoneal exudate cells to adhere to plastic provides an indicator of the number of macrophages in the peritoneal cavity and gives some indication of the animal's ability to respond to a stimulus and recruit this cell type. As was observed for the peritoneal cavity recruitability, TCE-2 or its vehicle produced no significant effects on the number of adherent cell populations in the female mice, and in the male mice, TCE-2 significantly depressed the number of adherent cells. However, no significant difference was found among the TCE-2 treatment groups, that is, there was no dose response (Figure 17). As with recruitability, the 1% emulphor exposure had no effect on cell adherence when compared to the distilled water group. It is difficult to explain why the males are more sensitive to this response than the females

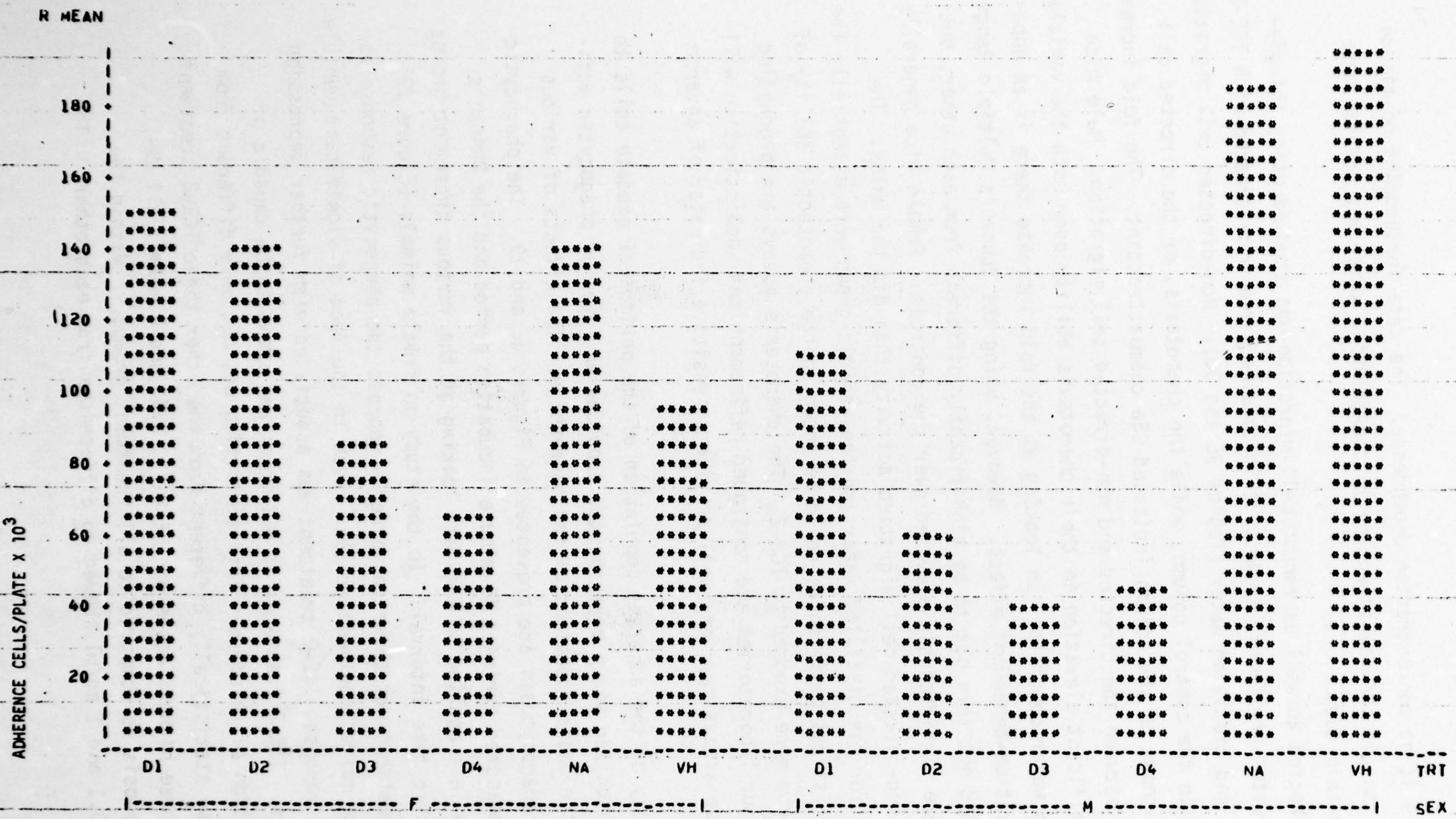
Table 34

Recruitability of Peritoneal Exudate Cells by Thioglycollate in Mice Exposed to Trichloroethylene

Group	Concentration in drinking water (mg/ml)	Males	Females
		x 10^6 cells/mouse	
Distilled water	—	38.5 ± 6.9	26.1 ± 3.3
1% Emulphor (vehicle)	—	50.4 ± 8.1	18.3 ± 3.6
TCE-2	0.1	28.7 ± 4.1*	24.2 ± 4.4
TCE-2	1.0	25.6 ± 3.8*	16.9 ± 2.4
TCE-2	2.5	18.3 ± 3.1*	22.6 ± 4.5
TCE-2	5.0	21.8 ± 2.5*	25.8 ± 6.8

Groups of CD-1 male and female mice were exposed to distilled water, 1% emulphor, or TCE-2 at 0.1, 1.0, 2.5, and 5.0 mg/ml for 120 days. Seven days prior to the end of the exposure period the mice were injected i.p. with 1.0 ml Brewers thioglycollate (10%). At the end of the exposure period, the peritoneal cavity was flushed with MEM and the number of cells recovered determined. Numbers represent the mean ± S.E. derived from 10 mice per group. *$p < .05$ as compared to vehicle control.

Figure 17
Adherence



D1 = 0.1 mg/ml TCE-2
D2 = 1.0 mg/ml TCE-2
D3 = 2.5 mg/ml TCE-2
D4 = 5.0 mg/ml TCE-2

and why there is no concentration dependence. The data forthcoming on tissue levels as a function of concentration in the drinking water may provide more insight into this data.

Cell-directed as well as random cell migration was studied using thioglycollate-recruited peritoneal exudate cells obtained from mice treated with TCE-2, the vehicle, and distilled water (Tables 35 and 36). Non-directed cell migration is indicated in the control column, while the chemotaxis, or the directed cell movement, is shown on the column indicated C5a chemoattractant. The fold increase is the ratio between the directed and non-directed cell migration. Male mice showed a significant elevation in their chemotaxis ability over both the vehicle and distilled water controls. In looking at the fold increase there is an apparent concentration-dependent effect. However, using the Duncan's Multiple Range Test, these did not turn out to be significantly different from each other, but again they were significantly elevated over the controls. Female mice generally had a lower, non-directed cell migration activity than did the males. The fold increase for the distilled water and the vehicle group were essentially the same. No statistical differences could be shown in the chemotactic ability of these cells from mice exposed to TCE-2. The chemotaxis assays are proceeding very well in our laboratories and continued refinement and sophistication will show that these assays may very well provide a sensitive indicator of adverse effects.

The ability of the adherent population of the peritoneal exudate cells to phagocytize opsonized sheep erythrocytes provides an index of phagocytic activity, one of the basic functions of the macrophage. The effects of various treatments on phagocytosis are presented in Figures 18 and 19. The phagocytic response was measured during a 45-minute incubation period and the Duncan's Multiple Range Test applied to the data looking at the various treatment groups across the entire time interval. In the study on female animals (Figure 19), the vehicle treatment was shown to markedly depress the phagocytic response as compared to the naive treatment groups. Even in the face of significant vehicle effect in this reponse, TCE-2 treatment was associated with further depression of phagocytosis. TCE-2 treatment at 0.1 mg/ml was shown to be capable of further inhibition of response. This inhibition was not only different from vehicle but also statistically different from the other three TCE-2 treatment groups. The three highest doses of TCE-2 (1, 2.5, and 5 mg/ml) all depressed phagocytosis as compared to all treatment groups. Within the TCE-2 groups, 0.1 and 1 mg/ml showed no differences from each other. The

Table 35

Chemotaxis of Thioglycollate-Recruited Peritoneal Exudate Cells in Female Mice Exposed to Trichloroethylene

Group	Concentration of TCE-2 (mg/ml)	Control	C5a Chemoattractant	Fold Increase
Distilled water	—	5.0 ± 0.8	12.8 ± 1.7	2.6
1% emulphor	—	7.1 ± 1.4	19.0 ± 2.3	2.7
TCE-2	0.1	4.9 ± 1.8	12.4 ± 1.9	2.5
TCE-2	1.0	6.4 ± 1.1	10.5 ± 1.5	1.6
TCE-2	2.5	6.7 ± 0.8	23.3 ± 3.2	3.5
TCE-2	5.0	2.8 ± 1.1	11.3 ± 2.8	4.0

CD-1 female mice were exposed to distilled water, 1% emulphor, or TCE-2 at indicated concentrations in the drinking water for 120 days. Peritoneal exudate cells were recruited with thioglycollate and chemotaxis performed in a Boyden chamber with and without the chemoattractant C5a. The numbers represent the mean ± S.E. derived from 10 mice per group.

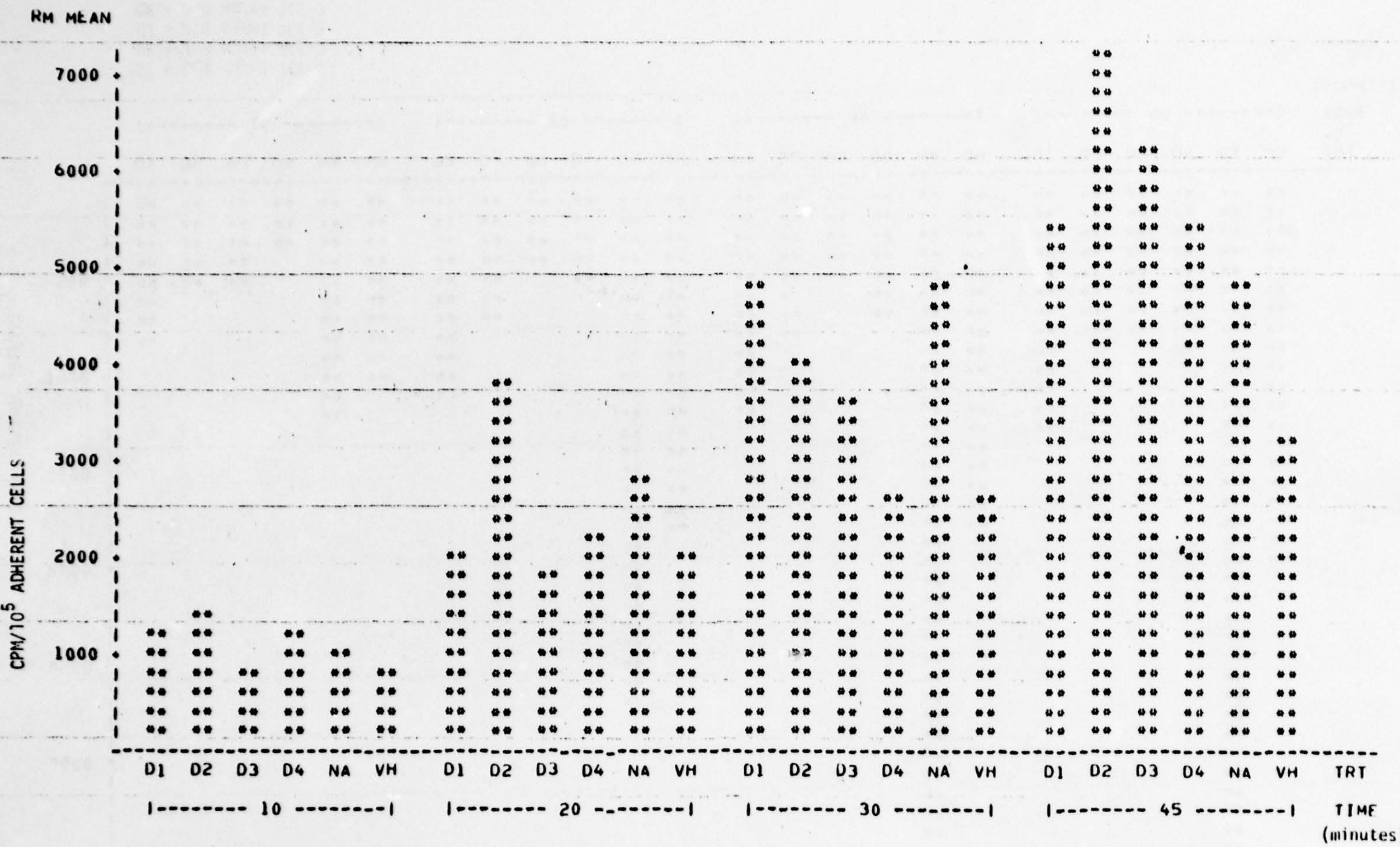
Table 36

Chemotaxis of Thioglycollate-Recruited Peritoneal Exudate Cells in Male Mice Exposed to Trichloroethylene

Group	Concentration of TCE-2 (mg/ml)	Control	C5a Chemoattractant	Fold Increase
Distilled water	—	8.4 ± 2.9	20.3 ± 3.2	2.4
1% emulphor	—	9.5 ± 2.3	15.0 ± 1.8	1.7
TCE-2	0.1	7.5 ± 3.0	24.1 ± 5.3	3.2*
TCE-2	1.0	7.7 ± 3.9	29.2 ± 2.2	3.8*
TCE-2	2.5	13.0 ± 4.3	58.3 ± 7.8	4.5*

CD-1 male mice were exposed to distilled water, 1% emulphor, or TCE-2 at indicated concentrations in the drinking water for 120 days. Peritoneal exudate cells were recruited with thioglycollate and chemotaxis performed in a Boyden chamber with and without the chemoattractant C5a. The numbers represent the mean ± S.E. derived from 10 mice per group.
* $P<.05$ compared to vehicle control.

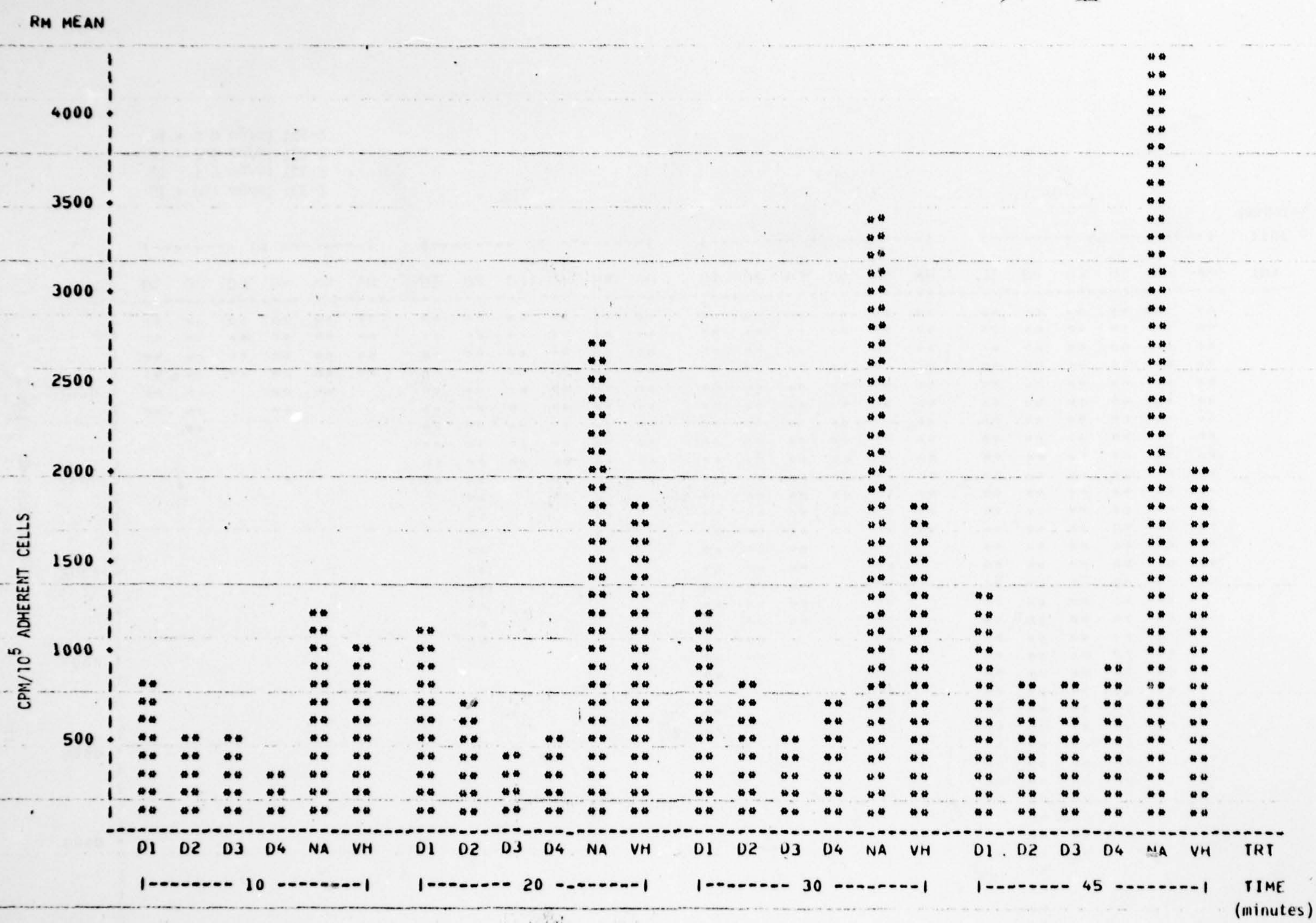
Figure 18
Phagocytosis - Males



D1 = 0.1 mg/ml TCE-2
D2 = 1.0 mg/ml TCE-2
D3 = 2.5 mg/ml TCE-2
D4 = 5.0 mg/ml TCE-2

Figure 19

Phagocytosis - Females



D1 = 0.1 mg/ml TCE-2
D2 = 1.0 mg/ml TCE-2
D3 = 2.5 mg/ml TCE-2
D4 = 5.0 mg/ml TCE-2

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DEVELOPMENT OF SHORT TERM IMMUNOTOXICOLOGICAL ASSAYS FOR THE PR--ETC(U)

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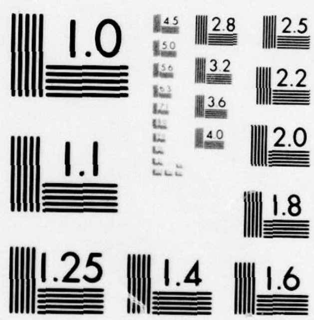
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interpretation of this data is very difficult because of the pronounced vehicle effect.

The results on recruitability, adherence, chemotaxis, and phagocytosis clearly attest to the complexity of evaluating a variety of responses utilizing a common population of cells. The relationship among the various parameters is at best speculative and very poorly understood. The main objectives of this particular study were to evaluate functions associated with the macrophage. The studies on the effect of TCE-2 on recruitability and adherence are probably better indices of bone marrow function than the adherent population size, though it is possible that they can also reflect more functional changes in these cells. Only further studies, both _in vivo_ and _in vitro_, will be able to explain the complexity of these responses and interactions. It is too early to make any meaningful interpretations of this data.

DEXAMETHASONE IN VIVO

Acute toxicity studies were performed on CD-1 male and female mice using a single gavage. These mice survived 1 g/kg dexamethasone. We have chosen not to give higher doses at this time because the information gained would not relate appreciably to the exposure by drinking water. Information provided by Merck, Sharpe, and Dohme indicated the LD_{50} to be about 600 mg/kg. We do not know how to reconcile this difference except to suggest that the mice were from different sources.

Subchronic 14-day range-finding experiments were set up whereby mice were exposed to dexamethasone in the drinking water. In the first experiment, 5 concontrations from 0.3 µg/ml to 10.0 µg/ml were used. Body weights were measured 5 times over the 2 week period and at the end of the exposure period organ weights and leukocyte counts were determined. Table 37 shows body weight data from mice weighed on days 0, 4, 8, 11, and 15. Mice exposed to 10.0 µg/ml began to demonstrate significant weight loss from days 8 through 15. Mice dosed at 2.5 µg/ml showed significant weight loss toward to end of the exposure period.

Table 38 represents data collected on the organ weights from these mice. The data is expressed as percent body weight. Spleen and thymus weights were significantly decreased as compared to controls in a dose response manner; the higher the dose of dexamethasone, the smaller the spleen and thymus. Surprisingly, no significant changes were observed in leukocyte counts.

A second study was performed using concentrations between 1.25 µg/ml and 0.1 µg/ml. Table 39 summarizes the data. The mice receiving 1.25 µg/ml in the drinking water did not gain weight at the same rate as the controls (Table 39). The only dexamethasone-induced organ weight change was a reduction in spleen weight. Thymic involution did not occur in this experiment at the 1.25 µg/ml level. In the first experiment, the thymic weight was decreased 26%. Based on the body and organ weight data, the concentration in the drinking water between 1.25 µg/ml and 2.5 µg/ml should produce effects. Table 40 shows the hematologic data on mice exposed to dexamethasone. There were no effects that were statistically different from controls. If the number of mice per group had been larger, a statistically significant decrease in leucocytes might have occurred. Suggestive data indicating changes in three coagulation factors are shown in Table 41. The number of mice per group is extremely small. However, data from a 90-day study where N = 10 or greater may

Table 37

Body Weights in CD-1 Female Mice
Exposed to Dexamethasone in the
Drinking Water over Fourteen Days

	Days				
Group	0	4	8	11	15
Vehicle	26.4 ± 0.7	28.2 ± 0.6	27.9 ± 0.6	28.8 ± 0.6	28.7 ± 0.5
0.3 μg/ml	27.4 ± 0.4	27.9 ± 0.6	28.7 ± 0.6	28.7 ± 0.5	27.9 ± 0.5
0.6 μg/ml	27.8 ± 0.5	28.9 ± 0.6	28.9 ± 0.6	29.1 ± 0.4	28.7 ± 0.4
1.25 μg/ml	28.0 ± 0.5	28.2 ± 0.5	27.8 ± 0.4	27.8 ± 0.4	27.2 ± 0.4
2.50 μg/ml	27.4 ± 1.1	27.2 ± 0.9	25.9 ± 1.0	26.0 ± 0.9	24.9 ± 1.0*
10.00 μg/ml	27.9 ± 0.5	27.3 ± 0.6	25.5 ± 0.6*	25.4 ± 0.6*	23.0 ± 0.8*

1) = 5 mice/group

2) data is represented as mean ± standard error

3) * = statistically different from vehicle control at $p < 0.05$

Table 38

Organ Weights as Percent of Body Weight and Leucocyte Counts on CD-1 Female Mice Exposed to Dexamethasone in the Drinking Water for Fourteen Days

Group	Spleen	Thymus	Leucocytes ($\times 10^3/mm^3$)
Vehicle	0.54 ± 0.02	0.26 ± 0.02	10.8 ± 1.3
1.3 μg/ml	0.56 ± 0.04	0.25 ± 0.01	12.7 ± 1.0
0.6 μg/ml	0.48 ± 0.02	0.22 ± 0.02	13.0 ± 0.8
1.25 μg/ml	0.39 ± 0.02*	0.19 ± 0.01*	9.8 ± 0.8
2.50 μg/ml	0.30 ± 0.02*	0.10 ± 0.01*	8.3 ± 1.0
10.00 μg/ml	0.19 ± 0.02*	0.10 ± 0.01*	7.7 ± 1.3

1) n = 5 mice/group

2) data is represented as mean ± standard error

3) * = statistically different from vehicle control at $p < 0.05$

Table 39

Body and Organ Weights of Weanling Female Mice Exposed for Fourteen Days to Dexamethasone in Drinking Water[a]

Treatment	No. Mice	Body Wt. (g)	Organ	Weight (mg)	% Body Weight	Organ/Brain
Vehicle[b]	5	25.8 ± .45	Brain	451.6 ± 13.0	1.75 ± 0.07	
			Liver	1321.4 ± 83.8	5.11 ± 0.28	2.95 ± 0.26
			Spleen	191.0 ± 19.5	0.74 ± 0.07	0.42 ± 0.05
			Lungs	195.6 ± 11.7	0.76 ± 0.05	0.43 ± 0.02
			Thymus	75.6 ± 6.0	0.29 ± 0.02	0.17 ± 0.01
			Kidneys	347.8 ± 15.4	1.35 ± 0.06	0.77 ± 0.03
0.1 μg/ml	5	25.0 ± .30	Brain	476.4 ± 10.2	1.90 ± 0.04	
			Liver	1253.0 ± 70.5	5.01 ± 0.33	2.64 ± 0.17
			Spleen	168.2 ± 17.1	0.67 ± 0.07	0.35 ± 0.03
			Lungs	209.6 ± 10.5	0.84 ± 0.05	0.44 ± 0.02
			Thymus	90.0 ± 7.7	0.36 ± 0.03	0.19 ± 0.02
			Kidneys	368.6 ± 18.5	1.47 ± 0.08	0.77 ± 0.03
0.3 μg/ml	5	25.6 ± .57	Brain	436.0 ± 6.0	1.70 ± 0.03	
			Liver	1341.4 ± 66.7	5.22 ± 0.22	3.08 ± 0.16
			Spleen	162.2 ± 17.2	0.63 ± 0.05	0.37 ± 0.04
			Lungs	187.0 ± 12.3	0.73 ± 0.04	0.43 ± 0.02
			Thymus	84.2 ± 9.3	0.33 ± 0.03	0.19 ± 0.02
			Kidneys	362.6 ± 19.0	1.41 ± 0.05	0.83 ± 0.03
0.6 μg/ml	5	23.9 ± .55	Brain	431.2 ± 16.0	1.81 ± 0.10	
			Liver	1254.4 ± 90.5	5.23 ± 0.27	2.95 ± 0.32
			Spleen	118.4 ± 17.7[c]	0.49 ± 0.07	0.28 ± 0.04[c]
			Lungs	161.6 ± 15.4	0.67 ± 0.06	0.38 ± 0.04
			Thymus	90.8 ± 9.3	0.38 ± 0.04	0.21 ± 0.03
			Kidneys	363.4 ± 10.0	1.52 ± 0.02	0.85 ± 0.05
1.25 μg/ml	5	22.9 ± .97[a]	Brain	443.8 ± 12.7	1.94 ± 0.07	
			Liver	1173.2 ± 42.6	5.13 ± 0.14	2.64 ± 0.06
			Spleen	119.2 ± 10.4[c]	0.53 ± 0.05	0.27 ± 0.02[c]
			Lungs	190.8 ± 12.8	0.83 ± 0.05	0.43 ± 0.02
			Thymus	62.8 ± 4.2	0.28 ± 0.02	0.14 ± 0.01
			Kidneys	341.6 ± 14.0	1.49 ± 0.05	0.77 ± 0.03

[a] Values represent mean ± SE

[b] Vehicle = tap water

[c] $P<0.05$ as calculated by Dunnett's T test

Table 40

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Hematological Parameters in Weanling Female Mice[a]

Treatment	Hematocrit (%)	Hemoglobin (g%)	Erythrocytes ($x10^6/mm^3$)	Leukocytes ($x10^3/mm^3$)	Platelets ($x10^5/mm^3$)
Vehicle[b]	40.6 ± 0.5 (5)[c]	12.6 ± 0.2 (5)	12.20 ± 0.68 (4)	9.59 ± 1.60 (4)	1.55 ± 0.13 (5)
0.1 µg/ml	41.0 ± 0.6 (3)	15.0 ± 2.0 (3)	13.43 ± 1.2 (3)	11.63 ± 1.60 (3)	1.66 ± 0.11 (3)
0.3 µg/ml	38.0 ± 1.4 (5)	11.0 ± 1.0 (5)	12.67 ± 1.2 (5)	7.50 ± 1.34 (5)	1.48 ± 0.22 (5)
0.6 µg/ml	41.4 ± 0.9 (5)	13.6 ± 0.3 (5)	13.68 ± 0.76 (5)	8.10 ± 0.49 (5)	1.92 ± 0.13 (5)
1.25 µg/ml	41.8 ± 0.6 (5)	11.7 ± 1.0 (5)	13.56 ± 1.1 (5)	6.16 ± 0.77 (5)	2.10 ± 0.29 (5)

[a] Values represent mean ± SE

[b] Vehicle = tap water

[c] ( ) = number of mice/group

Table 41

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Blood Coagulation in Weanling Female Mice[a]

Treatment	Fibrinogen (mg %)	Prothrombin Time (sec)	APTT[b] (sec)
Vehicle[c]	188.0 ± 27.0 (5)[d]	9.4 ± 0.2 (5)	28.1 ± 3.4 (4)
0.1 μg/ml	198.3 ± 7.3 (3)	9.3 ± 0.0 (3)	28.8 ± 1.8 (3)
0.3 μg/ml	166.3 ± 5.9 (4)	5.9 ± 0.2[e] (4)	24.0 ± 1.6 (4)
0.6 μg/ml	172.0 ± 4.1 (5)	7.8 ± 0.7[e] (4)	26.4 ± 1.1 (5)
1.25 μg/ml	242.5 ± 46.3 (4)	8.1 ± 0.1[e] (5)	63.6 ± 24.8[e] (2)

[a] Values represent mean ± SE

[b] Activated Partial Thromboplastin Time

[c] Vehicle = tap water

[d] ( ) = number of mice/group

[e] Significantly different from vehicle control group at $p<0.05$ by Dunnett's T test

provide a sensitive index for toxicity. Mice exposed to dexamethasone had a higher microsomal protein content per gram of liver (Tables 42 and 43). Mice exposed to 0.6 μg/ml and 1.25 μg/ml had a 34 and 49% increase in microsomal protein. No other parameters were altered.

Based on these two range finding studies, we are proposing that the concentration of dexamethasone in the drinking water for the 90-day study be 0.3 μg/ml, 0.6 μg/ml, 1.25 μg/ml, and 2.0 μg/ml. This study will provide a positive control for all the _in vitro_ studies and hopefully will provide a non-effect level and a pronounced effect level.

Table 42

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Hepatic Microsomal Hemoprotein Content in Weanling Female Mice[a]

Treatment	No. of Mice	Microsomal Protein (mg/g liver)	Cytochrome P450 (nmol/mg)[b]	Cytochrome b_5 (nmol/mg)[c]	Cytochrome P450 (nmol/g)[d]	Cytochrome b_5 (nmol/g)[e]
Vehicle[f]	5	20.6 ± 0.8	0.629 ± 0.071	0.516 ± 0.038	12.9 ± 1.1	10.6 ± 0.5
0.1 μg/ml	5	26.2 ± 2.0	0.598 ± 0.040	0.483 ± 0.015	15.6 ± 1.5	12.6 ± 0.7
0.3 μg/ml	5	26.6 ± 1.2	0.755 ± 0.029	0.464 ± 0.037	20.1 ± 1.3	12.3 ± 0.8
0.6 μg/ml	5	27.9 ± 1.9[g]	0.741 ± 0.071	0.412 ± 0.038	20.6 ± 2.2	11.5 ± 1.4
1.25 μg/ml	5	30.7 ± 1.2[g]	0.662 ± 0.090	0.417 ± 0.032	20.7 ± 3.6	12.9 ± 1.4

[a] Values represent mean ± SE

[b] nmol cytochrome P450/mg microsomal protein

[c] nmol cytochrome b_5/mg microsomal protein

[d] nmol cytochrome P450/g liver wet weight

[e] nmol cytochrome b_5/g liver wet weight

[f] Vehicle = tap water

[g] Significantly different from vehicle-treated group at $p<0.05$ as determined by Dunnett's T test.

Table 43

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Heptatic Microsomal Enzyme Activity and Glutathione Content in Weanling Female Mice[a]

Treatment	No. of Mice	Aniline Hydroxylase (nmol/mg/min)[b]	Aminopyrine N-Demethylase (nmol/mg/min)[c]	Aniline Hydroxylase (nmol/g/min)[d]	Aminopyrine N-Demethylase (nmol/g/min)[e]	Glutathione (μmol/g)[f]
Vehicle[g]	5	1.60 ± 0.16	11.30 ± 1.17	32.9 ± 2.1	231 ± 16	5.35 ± 0.3[illegible]
0.1 μg/ml	5	1.57 ± 0.11	9.98 ± 0.66	41.2 ± 4.0	259 ± 15	5.63 ± 0.25
0.3 μg/ml	5	1.50 ± 0.14	9.02 ± 0.69	39.8 ± 3.5	239 ± 19	6.09 ± 0.32
0.6 μg/ml	5	1.64 ± 0.03	8.74 ± 0.48	45.0 ± 3.1	242 ± 12	4.52 ± 0.12
1.25 μg/ml	5	1.56 ± 0.09	8.69 ± 1.06	48.0 ± 3.8	269 ± 39	5.28 ± 0.22

[a] Values represent mean ± SE

[b] nmol p-animophenol formed/mg microsomal protein

[c] nmol formaldehyde formed/mg microsomal protein

[d] nmol p-aminophenol formed/g liver wet weight

[e] nmol formaldehyde formed/ g liver wet wieght

[f] μmol reduced glutathione/g liver wet weight

[g] Vehicle = tap water

PART TWO - IN VITRO

DEVELOPMENT OF TIER ASSAYS

Rationale for In Vitro Exposure Method of Bone Marrow, Spleen, and Thymus Cells

The original experiments performed *in vitro* were done using the following procedure: Bone marrow single cell suspensions were made by flushing the mouse femur with media supplemented with 10% calf serum using a 23-gauge needle and a 10-ml syringe. Spleen and thymus single cell suspensions were prepared by pressing the organs through a wire mesh screen with media supplemented with 10% calf serum. Cell suspensions were then centrifuged at 250 g for 10 min. at 4° C. Cell concentrations were adjusted to 3-5 x 10^6/ml. One ml aliquots were added to individual sterile test tubes. To these tubes was added a 0.02 ml volume of the test chemical to obtain a desired molarity. Tubes were incubated at 37° C, 10% CO_2, 95% humidity for the desired time period and then removed for appropriate assay. The disadvantage of this procedure soon became evident. Large numbers of the tubes were required for each assay, and the time differences among the samples due to the time required to add the compound to each individual tube was introducing a large variable into each experiment. Also, using this exposure, we were only able to go as high as a 10^{-3}M exposure concentration.

We then modified the cell exposure procedure as follows: Single cell suspensions were prepared as described above. Solutions were prepared in large volumes in 250-ml sterile plastic erlenmeyer flasks. Preparing solutions in this manner allowed us to increase the exposure molarity of the test chemicals to 10^{-2}M. To these solutions was added a volume of cells to yield final cell concentrations of 3 x 10^6/ml. Each flask was incubated at 37° C, 10% CO_2, 95% humidity on a rocker platform. At 15 min., 1, 3, 24, and 48 hours, the flasks were removed, shaken vigorously, and 12 ml of the cell suspension were removed for neutral red dye uptake, DNA synthesis, and bone marrow stem cell growth assays. Cells were centrifuged at 250 g for 10 min. at 4° C to remove media containing the test chemical and were then resuspended in fresh media. The advantages of this exposure procedure were that final chemical molarities could be increased and that large batches of cells could be exposed at the same time so that at least three different assays could be performed on a batch of cells exposed under identical conditions. One disadvantage of this procedure was the increased likelihood of contamination. Since the flasks had to be entered so often to remove samples, the chances for bacterial contamination became a factor for concern. In two experiments using the exposure procedure, the experiment had to be terminated at 48 hours due to con-

tamination. In one experiment, it was terminated at 24 hours. The only other disadvantage of this procedure was that due to the constant opening of the flask, the chances of the loss of a volatile chemical were greatly increased, thus introducing another variable into the experiment.

Because of these disadvantages, we have made a slight modification in our approach. After the cells are in suspension, they are aliquoted into individual 50-ml sterile siliconized glass centrifuge tubes. These tubes are then placed in a roller drum in a 37°C, 10% CO_2, 95% humidity incubator. At 15 min., 1, 2, 3, 24, and 48 hours, two tubes are removed, centrifuged at 250 g for 10 min. at 4°C, and resuspended in fresh media to a final cell concentration of 3×10^6/ml. We feel that this modification in our exposure procedure will increase the efficiency of our experiments, decrease experimental error, and decrease the possibility of contamination.

Assessment of the Cytotoxic Ability of Chemicals

Various assays have been utilized to evaluate cytotoxic chemical activity on bone marrow, spleen, and thymus cells exposed *in vitro*. Procedures for all of the following assays are presented in the Appendix of this report in standard operating procedure format.

Trypan Blue Dye Exlusion Assay

Cells were exposed to trichloroethylene (TCE-2) and dexamethasone. At various times following exposure, samples were removed and trypan blue dye was added. Cells were then looked at under 100X magnification and cells which took up the dye were considered to determine the percentage of cells which were non-viable by dividing the number of cells that took up the dye by the total number of cells counted at a specific time point. After two experiments, it was observed that no significant change occurred when compared to vehicle controls. It was also observed that total cell numbers markedly decreased and that only a limited number of replicates could be done. It was after these observations that it was decided that trypan blue was not a sensitive indicator for addressing the problem of cytotoxicity.

Chromium-51 Release

Bone marrow, spleen, and thymus cells were radioactively tagged with sodium chromate-51. This radioactive label enters the cell's cytoplasm and is released upon lysis of the cell. In theory, this is a very straightforward assay. However, in reality, non-specific radioactive release from cells that had not lysed was so high that it made interpretation of chemical-treated cell release difficult. The results of one of five experiments using ^{51}Cr-release are represented in Table 44. In these experiments, significant increases in cell lysis were observed, but no pattern could be established from experiment to experiment. Also, release was not that strikingly high, as would be expected. It was concluded that some ^{51}Cr was binding to the cell membrane and not entering the cytoplasm, thus accounting for the high spontaneous release observed in control cells. Since results were highly variable and since non-specific release was so high, it was decided to discontinue the use of this assay as a means for measuring the cytotoxic effect of chemicals on these lymphoreticular cells.

Indium-111 Release

An attempt was made to radioactively tag cells with Indium-111 and subsequently

Table 44

The effect of Trichloroethylene (TCE-2) with Spleen, Thymus, and Bone Marrow Cells on Chromium-51 Release (Expressed as CPM's)

SPLEEN CELLS

		TCE-2 Molarity			
Time (Hours)	Vehicle	10^{-6}	10^{-5}	10^{-4}	10^{-3}
2	1234±3.7	956±72	765±30	892±105	1034±73
4	1245±61	1140±37	1230±92	1267±101	1417±73
8	1919±47	1410±125*	1658±162	1897±51	1709±67
16	1347±125	1248±41	1348±23	1286±55	1631±81*
24	1701±275	1333±56	1465±45	1579±223	1401±76
48	1575±27	1751±49	1560±71	1475±6	1849±179*

THYMUS

		TCE-2 Molarity			
Time (Hours)	Vehicle	10^{-6}	10^{-5}	10^{-4}	10^{-3}
2	486±6	415±10	424±5	520±35	569±42
4	948±41	757±30*	917±75	933±94	1999±933
8	1569±162	1173±26*	1419±75	1353±127	1268±46*
16	1347±125	1248±41	1348±23	1386±55	1631±81*
24	1701±275	1333±56	1465±45	1579±223	1401±76
48	1565±27	1751±49	1560±71	1475±6	1849±179*

BONE MARROW CELLS

		TCE-2 Molarity			
Time (Hours	Vehicle	10^{-6}	10^{-5}	10^{-4}	10^{-3}
2	506±139	429±58	414±58	609±243	613±135
4	505±38	526±62	578±88	785±354	593±163
8	944±125	1266±215	1403±146	766±27	998±132
16	1748±75	757±38*	1321±83	724±100*	1751±88
24	899±34	1643±267*	1004±62	797±80	680±61*
48	801±109	912±55	941±66	1772±105*	1459±212*
72	1436±133	927±40	2504±327*	1810±193	2009±88*

1. These numbers represent ^{51}Cr released (expressed as CPM's) by chromated cells after exposure to the compound.
2. All data is represented as the mean± S.E. of 3 microtiter wells.
   * p <.05

measure cell lysis by the release of Indium-111. Once again, spontaneous release was high and interpretation of results was difficult. Three experiments were performed and the results are represented in Table 45. Release in cells treated with TCE-2 and dexamethasone at 10^{-2}M are only slightly above that of appropriate vehicle controls and no pattern could be established from experiment to experiment. Due to the high variability among experiments and the high spontaneous release observed, this assay was discontinued as a means of measuring cytotoxicity.

Neutral Red Dye Uptake

In this assay, cells are exposed to neutral red dye which will only be taken up by cells which are viable and functioning properly. Our studies thus far in development of this assay provide positive signs that it may be more reproducible, sensitive, and inexpensive. This procedure is still in the developing stages since we are experimenting with methods to increase the sensitivity of the assay. Typical results obtained from preliminary experiments using this method are contained in Table 46.

Table 45

Indium-111 Release From Spleen Cells
Exposed *In Vitro* to Trichloroethylene

CPM'S

		TIME (HOURS)						
		0	2	8	18	24	40	48
Experiment I	Control	1271 ± 126	1217 ± 117	2404 ± 113	2404 ± 113	2421 ± 111	2436 ± 269	2331 ± 106
	TCE 10^{-2}M	947 ± 133	1679 ± 172	2263 ± 173	2325 ± 95	2829 ± 593	2369 ± 259	2260 ± 243
	Dexa 10^{-2}M	1647 ± 49	1683 ± 139	2325 ± 95	2263 ± 173	2359 ± 94	2362 ± 256	2261 ± 170
Experiment II	Control	10620 ± 913	8774 ± 361	9230 ± 299	9810 ± 479	9759 ± 782	8912 ± 420	±
	TCE 10^{-2}M	14261 ± 811	11600 ± 764	12470 ± 658	12863 ± 235	11996 ± 918	11219 ± 869	±
	Dexa 10^{-2}M	15858 ± 595	12516 ± 911	12271 ± 557	13809 ± 936	12780 ± 654	12480 ± 301	±
Experiment III	Control	62214 ±4614	22152 ±3086	55903 ±2019	67597 ±1410	64442 ±2066	59233 ±2974	35541 ±2311
	TCE 10^{-2}M	25255 ±1522	23444 ± 912	26421 ±1409	26012 ± 840	25000 ± 799	22320 ±2006	11560 ±1008
	Dexa 10^{-2}M	28093 ±1116	10433 ±1018	19596 ±1495	23357 ±1146	22598 ±1700	21327 ±2664	13480 ±1181

1) n = 8 microtiter wells/concentration/time point

2) All data is represented as mean ± standard error.

Table 46

Neutral Red Dye Uptake in Bone Marrow Cells
Exposed *In Vitro* to Trichloroethylene

μg neutral red dye extracted

		TIME				
		15 min	1 hr	3 hr	24 hr	48 hr
Experiment I	Control		20.9 ± 0.4	25.1 ± 0.4	27.0 ± 1.0	28.7 ± 3.1
	TCE 10^{-2}M		18.3 ± 0.8*	19.8 ± 0.7*	23.6 ± 0.8*	22.0 ± 0.6*
Experiment II	Control	13.6 ± 0.8	13.9 ± 1.4	12.3 ± 0.2	11.0 ± 0.6	10.8 ± 0.6
	TCE 10^{-4}M	14.4 ± 0.9	13.8 ± 0.6	12.3 ± 0.5	10.5 ± 0.8	11.6 ± 0.5
	TCE 10^{-3}M	13.1 ± 0.7	14.1 ± 0.4	12.7 ± 0.7	10.5 ± 0.5	11.2 ± 0.6
	TCE $10^{-2.5}$M	13.9± 0.9	14.5 ± 0.3	12.2 ± 0.9	10.8 ± 0.8	11.7 ± 0.3
	TCE 10^{-2}M	8.8 ± 0.6*	9.9 ± 0.9*	8.9 ± 0.3*	9.6 ± 0.4	11.2 ± 0.9
Experiment III	Control	6.7 ± 1.5	5.1 ± 0.6	3.5 ± 0.4	3.4 ± 1.0	4.0 ± 0.8
	TCE 10^{-4}M	6.4 ± 0.8	5.3 ± 0.9	3.2 ± 1.1	4.0 ± 0.9	11.2 ± 0.2*
	TCE 10^{-3}M	6.4 ± 1.3	4.8 ± 1.3	4.2 ± 0.9	2.8 ± 0.7	8.0 ± 1.8*
	TCE $10^{-2.5}$M	5.0 ± 0.7	4.6 ± 0.9	2.1 ± 0.6	1.2 ± 0.7	2.8 ± 0.5
	TCE 10^{-2}M	1.2 ± 0.4*	3.1 ± 0.2*	0.3 ± 0.4*	0.6 ± 0.6*	5.1 ± 0.1

1) n = 4-6 tubes/concentration/time point.
2) All data is represented as mean ± standard error.
3) * = statistically different from vehicle controls as determined by Dunnett's T-Test.

DNA Synthesis Studies In Vitro

These studies are included in the Tier One assays and assess the ability of a chemical to interfere with the incorporation of iododeoxyuridine (IUdR) into DNA. We have selected IUdR instead of thymidine because of the gamma emitter ^{125}I which labels IUdR. By using a gamma emitter, the liquid scintillation expense and time are markedly reduced.

Prior to using ^{125}I-IUdR as an indicator of DNA synthesis, we determined that the IUdR was incorporated into DNA. Results of this experiment are shown in Table 47. Five x 10^6 bone marrow cells were labelled with ^{125}I-IUdR for 3 hours. This was followed by precipitation with 10% trichloroacetic acid and collection on filter paper. The supernatant from these cells was also collected. An aliquot of labelled cells was acid hydrolyzed at 95°C for 30 minutes to solubilize the DNA. The supernatant from this sample contained soluble DNA and the remaining pellet DNA free. In order to show that IUdR did not incorporate into RNA, TCA-precipitated cells were washed and resuspended in 0.3 NaOH and incubated at 37° C for 1 hour. Hepes buffer was used for neutralization. Four ml of 1.6 M TCA were added to the cells, centrifuged, and samples of the supernatant and pellet were collected and analyzed for ^{125}I. The data provided in Table 47 shows that only the acid precipitable material contains ^{125}I-IUdR. Thus we feel confident that IUdR will provide an indicator of DNA synthesis.

Several experiments were performed to determine the potential effects of TCE-2 and dexamethasone on DNA synthesis (Tables 48 and 49). The chemicals were added directly to the microtiter wells; in test tubes, cells were pre- and post-exposed to the chemical with respect to IUdR pulse. Non-stimulated and in vivo stimulated (with methyl vinyl ether polymer or bacterial lipopolysaccharide) spleen cells were investigated. Pulse times and the amount of ^{125}I-IUdR were studied along with the presence or absence of fluorodeoxyuridine (Figure 20). Fluorodeoxyuridine increases the incorporation of IUdR into DNA of certain cells supposedly by inhibition of thymidylate synthetase which effectively blocks thymidine incorporation into DNA. Thus thymidine does not act as a competitor for IUdR incorporation. Bone marrow cells incoporate IUdR at a much higher level in the presence of FUdR, while FUdR does not markedly affect the amount of IUdR incoporated into spleen cells. Thus we are presently using FUdR for bone marrow cells, but not for spleen cell experiments.

Generally, DNA synthesis in bone marrow cells and spleen cells decreases to zero after 24 hours in culture. We believe this does not represent a loss of cell

Table 47

Evidence That Iododeoxyuridine is Incorporated Into DNA

Treatment	CPM/10^5 Cells	Treatment	CPM/10^5 Cells
cells Before Acid Hydrolysis	2969 ± 55	cells Before Base Hydrolysis	2833 ± 93
Supernatant After Acid Hydrolysis	3053 ± 22	Supernatant After Base Hydrolysis	132 ± 8
Precipitate After Acid Hydrolysis	158 ± 6	Precipitate After Base Hydrolysis	1353 ± 27

3×10^6 cells/ml incubated, with shaking, for 2 hrs. at 37°C with IUdR (0.1 μCi/20λ). Some cells were precipitated with cold 10% TCA and samples were collected on cell harvester. An aliquot of labelled cells with 10% TCA was hydrolyzed at 95°C for 30 mins to solubilize DNA. The supernatant containing DNA was counted, while the remaining pellet (DNA-free) was collected on the cell harvester. Still another aliquot of labelled cells was neutralized by adding Hepes Buffer before precipitation with 10% TCA. Samples were collected on cell harvester. The cells were resuspended in 0.3N NaOH and incubated for 1hr. at 37°C. Again, the suspension was neutralized with Hepes and 1.6M TCA was added. The supernatant contained soluble RNA and the pellet contained DNA.

Table 48

Incorporation of ^{125}I-IUdR in Mouse Bone Marrow Cells Exposed *In Vitro* to Trichloroethylene and Dexamethasone

	CPM/10^5 Cells (Mean±S.E.)			
	1 hr.	2 hr.	3 hr.	4 hr.
Vehicle	2690±36	4363±170	5392±40	4865±134
Dex 10^{-3}	1846±89*	3824±64*	4380±227*	4285±70*
Dex 10^{-2}	353±41*	1737±78*	1900±95*	2320±83*
EtOH Vehicle	2543±124	4598±132	5538±42	4878±164
TCE 10^{-3}	2429±56	2985±140*	4192±80*	4672±160
TCE 10^{-2}	44±8*	54±7*	32±2*	103±38*
Ara-C 10^{-4}				134±49

1. Bone marrow cells adjusted to 3×10^6 cells/ml in each dose of drug.
2. Bone marrow cells incubated 15 minutes with the drug.
3. IUdR - 0.1 u CI/20λ per well in microtiter plates.
4. Incubated 1, 2, 3, 4 hours. Harvested on cell collector.
5. n = 6 microtiter wells/data point

* p <.05 as compared to vehicle control.

Table 49

Incorporation of ^{125}I-IUdR in Mouse Bone Marrow Cells Exposed *In Vitro* to Trichloroethylene and Dexamethasone

	CMP/10^5 Cells					
	15 min.	30 min.	1 hr.	2 hr.	3 hr.	4 hr.
Vehicle	444±63	920±81	1746±87	3366±208	3605±161	2202±158
Dex 10^{-4}	455±39	792±57	1494±101*	3093±157	3597±179	3759±70*
Dex 10^{-3}	612±47*	937±44	1663±113	2961±104*	3514±170	2869±83*
Dex $10^{-2.5}$	544±34	827±51	1290±69*	2916±58*	2695±210 *	2183±149
Dex 10^{-2}	286±30*	490±25*	969±62*	1753±123*	2293±52*	1955±33
EtOH Vehicle	918±50	1252±29	1581±144	3722±49	4227±240	4230±221
TCE 10^{-4}	611±52*	1056±53*	1719±118	3787±94	4305±158	4425±194
TCE 10^{-3}	715±32*	991±52*	2035±76*	2918±147*	3984±368	3185±254*
TCE $10^{-2.5}$	556±19*	739±52*	1168±171*	2648±242*	3426±264*	no sample
TCE 10^{-2}	43±15*	32±2*	46±22*	56±17*	58±26*	70±26*
Ara-C 10^{-4}			36±9			

n = 6 microtiter/data point.

* p <.05 as compared to vehicle control.

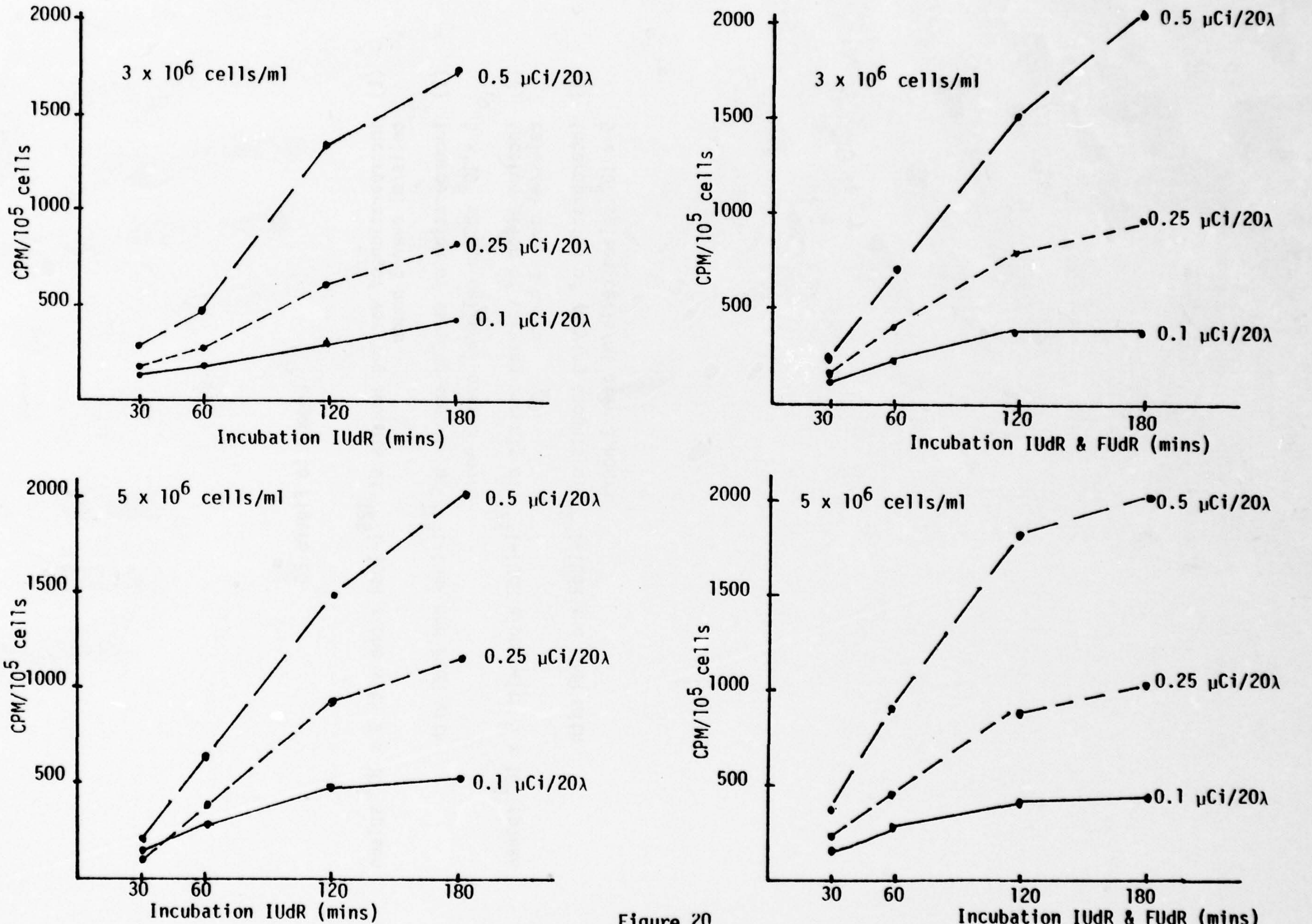


Figure 20
Incorporation of ^{125}I-IUdR into Spleen Cells:
Relationship to IUdR Concentration, Cell Number, and Presence of FUdR

Legend to Figure 20

(1) incorporation of varying amounts of ^{125}I-IUdR alone with 3×10^6 spleen cells/ml over 3 hours

(2) incorporation of varying amounts of ^{125}I-IUdR and FUdR with 3×10^6 spleen cells/ml over 3 hours

(3) incorporation of varying amounts of ^{125}I-IUdR alone with 5×10^6 spleen cells/ml over 3 hours

(4) incorporation of varying amounts of ^{125}I-IUdR and FUdR with 5×10^6 spleen cells/ml over 3 hours

viability, since bone marrow stem cell growth remains constant over the 48-hour incubation time course.

Data on the present status of DNA synthesis is provided in the section entitiled In Vitro Combined Experiments.

Bone Marrow Stem Cell Colony Formation

This assay represents a Tier Three assay and developed more rapidly than expected and has provided a potential sensitive target which may predict toxicity *in vivo*.

Preliminary experiments were done to determine what effect, if any, TCE-2 and dexamethasone had on the ability of bone marrow stem cells to form colonies *in vitro*. Single cell suspensions of bone marrow cells were exposed to TCE-2 or dexamethasone at varying concentrations under the designated conditions for 1 and 3 or 24 hours. The cell suspensions were washed to remove any residual chemicals in the media, and the resulting cell suspension was resuspended in 1.8% methyl cellulose in α-MEM. At the end of the seven day incubation period, using colony counts/10^5 cells plated as a criterion for comparison, it was observed that with an increasing concentration of either chemical there was a decrease in number of colonies formed (Tables 50 and 51).

Although these results showed an effect from both chemicals,the numbers of colonies/10^5 cells in the vehicle groups were well below previously established levels of about 150 colonies/10^5 cells plated out. This brought up the possibility that the exact number of cells plated out played an important part in determining the number of colonies formed. An experiment was designed to evaluate the effects of cell number and to hopefully discover an optimal cell number to use in future studies.

A cell suspension was made and diluted to various cell concentrations ranging from 1×10^7 cells/ml to 5×10^4 cells/ml. These cell suspensions were further diluted in methyl cellulose (as per the standard operating procedure), and plated out. After the seven day incubation, it was observed that cell density did affect colony formation (Table 52). Adjustment of all future cell counts will be in the ranae of 0.5×10^6/ml and 1.0×10^6/ml.

Experiments were performed to repeat the preliminary studies, with the cell counts adjusted to the optimum range. The same trend in chemical effects was observed and the colony numbers were higher and more reproducible (Table 52). A dose-dependent suppression of bone marrow stem cell formation was seen with both dexamethasone and TCE-2. In the case of TCE-2, significant inhibition was seen (60%) at 10^{-5}M while it required 10^{-3} dexamethasone to produce a significant (40%) reduction (Tables 50 and 51).

Table 50

Effect of TCE-2 on Bone Marrow Stem Cells

Group	cell counts after exposure / ml	colonies / 10^5 cells
Vehicle	1.0×10^6	154.0 ± 4.4
10^{-5}M	2.4×10^6	$61.9 \pm 4.8^*$
10^{-4}M	3.9×10^6	$28.9 \pm 4.5^*$
10^{-3}M	4.5×10^6	$27.6 \pm 3.4^*$
10^{-2}M	7.7×10^6	$16.8 \pm 0.7^*$

Bone marrow cells were incubated with TCE-2 in concentrations indicated for 24 hours. The number of stem cells were determined by colony formation. Numbers represent the mean ± S.E. derived from 6 cultures.

* $p < .05$ as compared to vehicle.

Table 51

Effect of Dexamethasone on Bone Marrow Stem Cells

Group	cell counts after exposure / ml	colonies / 10^5 cells
Vehicle	1.5×10^6	101.7 ± 4.1
10^{-4}M	1.5×10^6	100.3 ± 6.2
10^{-3}M	2.1×10^6	$59.4 \pm 3.3^*$
$10^{-2.5}$M	3.0×10^6	$29.3 \pm 3.5^*$
10^{-2}M	3.1×10^6	$30.3 \pm 3.3^*$

Bone marrow cells were incubated with dexamethasone in concentrations indicated for 24 hours. The number of stem cells were determined by colony formation. Numbers represent the mean ± S.E. derived from 6 cultures.

* $p < .05$ as compared to vehicle.

Table 52

Effect of Bone Marrow Cell Density on Colony Formation

Cells added	Experiment 1	Experiment 2
	colonies/10^5 cells	colonies/10^5 cells
5×10^4	26.7 ± 6.7	30.0 ± 5.8
1×10^5	35.0 ± 7.6	43.3 ± 6.0
5×10^5	213.3 ± 6.4	210.0 ± 6.4
7.5×10^5	—	237.6 ± 5.3
1×10^6	214.5 ± 1.9	212.3 ± 1.5
5×10^6	44.2 ± 0.9	43.9 ± 0.6
1×10^7	19.4 ± 0.3	20.1 ± 0.5

Mean ± standard errors derived from 3 cultures per cell concentration.

Experiments are being designed at this time to use glass tubes and flasks during exposure times and to increase the number of tubes (or flasks), thereby decreasing experimental error.

The bone marrow stem cell assay is developed the furthest of the Tier 3 assays. We will continue to refine this assay over the next year.

Phagocytosis

Presently, we are considering phagocytosis of opsonized sheep erythrocytes a Tier One assay. This assay is well established and is directed by Dr. R. Carchman. Thioglycollate-recruited peritoneal exudate cells were allowed to adhere for 18 hours prior to use. Following this period of adherence, the cells were exposed to TCE-2, dexamethasone, or drug vehicles. Representative data from these studies which was observed was a small ($\sim$30%), but significant ($p < 0.05$) depression in the dexamethasone-treated group (Table 53). This effect was not observed in the replicate study. We will extend the period of exposure of these cells to TCE-2 and dexamethasone in order to optimize the detection of any effects on phagocytosis.

The standard operating procedure for this assay is provided in the Appendix. It is performed as outlined in the original proposal and in the renewal proposal.

Table 53

^{51}Cr-SRBC Phagocytosis in Thioglycolate Stimulated Macrophages Exposed to TCE-2 and Dexamethasone

	% of Control Phagocytosis		
	Time Following ^{51}Cr-SRBC Addition		
	10	20	30
Control	100 ± 7	100 ± 6	100 ± 6
TCE-2 10^{-3}M	98 ± 2	100 ± 8	99 ± 5
10^{-4}M	102	114 ± 13	100
10^{15}M	103	107	108
Control	100 ± 8	100 ± 6	100 ± 7
DEXA 10^{-3}M	69 ± 4*	83 ± 7*	82 ± 6*
10^{-4}M	106	92 ± 10	129 ± 11
10^{-5}M	81 ± 17	83 ± 17	93 ± 21

Data is representative of 8 microtiter wells/time point.

*Statistically different from vehicle control at $p < 0.05$ as determined by Dannett's T Test.

Lymphocyte Responsiveness to Mitogens

This is a Tier Two assay which took longer to get off the ground than expected. It was important to use ^{125}I-IUdR instead of ^{3}H thymidine to measure lymphocyte blastogenesis because of the experimental size and the costs involved. Problems occurred with obtaining reproducible (experiment to experiment) results with the T cell mitogen phytohemagglutitin (PHA) and the appropriate bacterial lipopolysaccharide; the B-cell mitogen was not available for several months. It remains in short supply. Recently, a switch, suggested by a consultant to this project, from PHA to Conconavalin A, moved this aspect of the project along.

The following data and comments provide a status report of lymphocyte responsiveness to T and B lymphocyte mitogens.

Effect of Fluorodeoxyuridine on the Uptake of ^{125}I-Iododeoxyuridine

Fluorodeoxyuridine is necessary for the uptake of ^{125}I-iododeoxyuridine. These experiments were designed to determine the relationship between the concentration of FUdR on IUdR uptake. As indicated in earlier reports, employing ^{125}I-IUdR for measurement of DNA synthesis will allow more samples to be analyzed more rapidly at a lower expense because of the gamma emission. Spleen cells from five CD-1 mice were separated into single cell suspension by passage through a 100-mesh stainless steel screen using RPMI 1640 media. The spleen cells were washed and reconstituted to 2 ml with RPMI 1640 media containing 10% serum after cell counts were performed. The cell concentration was made up to 5×10^{6}/ml. One hundred μl, or 5×10^{5} spleen cells in RPMI 1640 were added to the wells of the microtiter plates. In the experiment shown in Table 54, 10% heat-inactivated fetal calf serum was used as the serum source. In the experiment shown in Table 55, heat-inactivated calf serum was employed. Calf serum was used because fetal calf serum is becoming extremely scarce and expensive. Thus, we believe that alternative sources of protein should be looked for to assure availability and to minimize the expense of the assay. Conconavalin A in concentrations of 0, 1, 5, and 10 μg/well was added as the T cell mitogen. The cultures were incubated at 37°C, 5% CO_2, and 95% relative humidity for 48 hours. At the end of the 48 hours, the cultures were pulsed with 50 μl of RPMI 1640 media containing 2 μCi of ^{125}I-iododeoxyuridine and fluorodeoxyuridine at the indicated concentrations. The isotope pulse time was set at 18 hours under the same incubation conditions described above. The cells were harvested onto filter paper using the Skatron cell harvester and radioassayed. The results are shown in Tables 54 and 55.

Table 54

Effect of Fluorodeoxyuridine Concentration on the Uptake of ^{125}I-Iododeoxyuridine into DNA of Spleen Cells Stimulated with Conconavalin A

(Fetal Calf Serum)

FUDR Concentration	Conconavalin A μg/well 0	1	5	10
5×10^{-4}M	2315 ± 275	30854 ± 806	28126 ± 604	4967 ± 357
1×10^{-4}M	4157 ± 146	62897 ± 2157	61009 ± 5042	6773 ± 251
5×10^{-5}M	4605 ± 125	88549 ± 7532	95419 ± 2071	11339 ± 338
1×10^{-5}M	5305 ± 230	155444 ± 3913	146357 ± 7886	14352 ± 655
5×10^{-6}M	4712 ± 150	163005 ± 2891	160027 ± 3977	16887 ± 501
1×10^{-6}M	4533 ± 220	169848 ± 5136	187839 ± 3901	22954 ± 1186

Mean ± standard errors derived from 6 replicate cultures.

Table 55

Effect of Fluorodeoxyuridine Concentration on the Uptake of ^{125}I-Iododeoxyuridine into DNA of Spleen Cells Stimulated with Conconavalin A

(Calf Serum)

FUDR Concentration	Conconavalin A μg/well 0	1	5	10
1×10^{-6}M	882 ± 109	535 ± 55	22177 ± 2390	64851 ± 2090
7.5×10^{-7}M	400 ± 35	563 ± 67	17742 ± 1572	56368 ± 2084
5.0×10^{-7}M	521 ± 57	354 ± 45	22548 ± 860	71170 ± 2964
2.5×10^{-7}M	817 ± 65	732 ± 86	18858 ± 1557	56644 ± 3190
1.0×10^{-7}M	535 ± 41	674 ± 56	17868 ± 680	62653 ± 2540
5.0×10^{-8}M	578 ± 47	490 ± 54	20963 ± 1201	68741 ± 5082
1.0×10^{-8}M	393 ± 32	528 ± 96	16875 ± 1050	75348 ± 3269
0	422 ± 41	502 ± 97	721 ± 111	662 ± 104

Mean ± standard error derived from 6 replicate cultures.

With fetal calf serum, the background response is between 2315 and 5305 cpm/culture. Peak response is seen at both the 1 and 5 μg/Conconavalin A per culture. There is an inverse concentration-dependent enhancement of ^{125}I-iododeoxyuridine uptake by fluorodeoxyuridine with maximum uptake occuring at 1×10^{-6}M.

The FUdR concentration study was continued (Table 55), but using calf serum in place of fetal calf. The background response is lower (393-882 cpm/culture). Peak response was shifted to 10 μg/culture. There was essentially no difference in uptake of ^{125}I-IUdR through 1×10^{-8}M.

Comparison of Two Bacterial Lipopolysaccharide Preparations

Bacterial lipopolysaccharides (LPS) are specific T lymphocyte mitogens for the mouse. We investigated two LPS preparations for their mitogenic potential. They were Westphal preparations and included LPS from S. typhosa and E. coli 011-b8. Spleen cells from five CD-1 mice were prepared into single cell suspensions in RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum. After cell counts were performed, the cell number was adjusted to 5×10^6 cells/ml. One hundred μl (5×10^5) spleen cells were added to the microtiter wells. Fifty μl of the LPS preparation in the indicated amounts were added to the microtiter wells. The cultures were incubated for 48 hours at 37° C, 5% CO_2, and 95% humidity. After 48 hours, 50 μl of RPMI 1640 media containing 0.2 μCi of ^{125}I-IUdR and FUdR were added in an amount to give a final concentration of 10^{-6}M in the culture. The 18-hour pulse period was carried out under the same incubation conditions, after which the cultures were harvested onto filter paper using a Skatron cell harvester and radioassayed in a Beckman scintillation counter. Table 56 shows the results of this experiment. Blastogenesis was not marked at any of the concentrations. Background activity was within normal limits, i.e. 700-1200 cpm/culture. Peak response occurred with 30 μg/well for both LPS preparations. In the case of 011-b8, the response was decreased 2.38-fold at the 50 μg/well from the peak response. This was not the situation with 0901.

Since this experiment was completed, we obtained LPS 011-b4 (Westphal) preparation which is reported to be a good B lymphocyte mitogen.

Comparison of Frozen and Non-Frozen Mitogens

Because of the large number of assays that will be performed in one day, a study was performed to determine if the mitogens could be prepared and frozen in

Table 56

Comparison of Two Bacterial Lipopolysaccharide Preparations for Mitogenic Activity

Concentration of LPS μg/well	LPS S. typhosa 0901 (Westphal)	LPS E. Coli 011-b8 (Westphal)
0	729 ± 39	1174 ± 123
0.5	4795 ± 132	3931 ± 713
1.0	5346 ± 581	4390 ± 416
5.0	4125 ± 472	5046 ± 295
10.0	5817 ± 570	5302 ± 236
30.0	7338 ± 407	8640 ± 1939
50.0	6548 ± 99	3628 ± 152

Mean ± standard error derived from 6 replicate cultures.

the microtiter plates so they would be ready on the day of the experiment.

Conconavalin A and bacterial lipopolysaccharide were prepared in the following concentrations: 2 μg/ml, 10 μg/ml, 20 μg/ml, 10 μg/ml, 200 μg/ml, 600 μg/ml, and 1 mg/ml. For each mitogen, 50 μl of each concentration was added to the 6 wells of a microtiter plate and the plates were maintained at -70° C until use. For comparison, similar microtiter plates (not frozen) were set up on the day the spleen cells were added. The plates were removed from the freezer and 200 μl of spleen cells containing 5 x 10^5 cells were added to the plates. The microtiter plates were incubated for 48 hours at 37° C and 95% relative humidity. Fifty μl of RPMI 1640 media containing 0.2 μCi ^{125}I-iododeoxyuridine and fluorodeoxyuridine (to give a final concentration of 10^{-6}M) was added to each well after 48 hours. After an 18 hour pulse with ^{125}I-IUdR, the spleen cells were harvested using a Skatron cell harvester. The filter paper containing the cells was radioassayed for ^{125}I in a Beckman 300 model Scintillation Counter. Table 57 shows the results which indicate that the microtiter plates can be prepared with the mitogen and frozen for future use.

The spleen mitogenicity experiments are progressing fairly well. We still have experiments where we obtain low incorporation of ^{125}I-IUdR. Continued studies are being performed to refine this assay and make it highly reproducible.

Table 57

Comparison of Spleen Cell Blastogenesis Response
Using Mitogens That Were Frozen and Non-Frozen

Mitogen		Concentration (µg/well)		Mitogen Prepared on Day of Use
	N	0	1095 ± 99	861 ± 140
Conconavalin A	6	0.1	N.D.	7919 ± 1087
	6	0.5	103272 ± 7652	109209 ± 6469
	6	1.0	126894 ± 9449	96739 ± 4718
	6	5.0	154866 ± 14981	157929 ± 10079
	6	10.0	6796 ± 1541	7985 ± 522
	6	30.0	1616 ± 327	2303 ± 965
	6	50.0	1089 ± 327	1282 ± 346
Lipopolysaccharide *S. typhosa* 0901	6	0.5	3739 ± 175	4795 ± 132
	6	1.0	4860 ± 239	5346 ± 581
	6	5.0	6212 ± 688	4125 ± 472
	6	10.0	6273 ± 283	5817 ± 570
	6	30.0	6558 ± 381	7338 ± 407
	6	50.0	6632 ± 147	6548 ± 99

Means ± standard error derived from 6 microtiter wells.

IN VITRO COMBINED EXPERIMENTS

We have performed several experiments using a common exposure of the bone marrow cells and performing different assays. The following represents a summary of the results.

Experimental Design

In order to better correlate the effects of the chemical and the tier assays, a combined approach to exposure and assay was attempted. Five experiments have been performed where the target cells, primarily bone marrow cells, were exposed to the chemical in flasks and samples removed for each assay at selected time intervals over 48 hours. For the last three experiments the following experimental design was followed:

Flask A - Vehicle
Flask B - Vehicle
Flask C - Vehicle
Flask D - 10^{-4}M trichloroethylene or dexamethasone
Flask E - 10^{-3}M trichloroethylene or dexamethasone
Flask F - $10^{-2.5}$M trichloroethylene or dexamethasone
Flask G - 10^{-2}M trichloroethylene or dexamethasone
Flask H - 10^{-2}M trichloroethylene or dexamethasone
Flask I - 10^{-2}M trichloroethylene or dexamethasone

Four variables are being investigated: (i) cell count, (ii) neutral red uptake, (iii) DNA synthesis, (iv) bone marrow stem cell growth. For each cell count there are triplicate determinations, for neutral red there are quadruplicate determinations, for DNA synthesis there are six replicates, and for bone marrow stem cell growth there are triplicate determinations. Five time intervals are being studied: 15 minutes, 1 hour, 3 hours, 24 hours, and 48 hours. For each variable, interflask effect, intra-flask effect, and dose effect will be investigated using analysis of variance, involving both crossed and nested classification with sample nested within the flask.

General Statistical Approach

For TCE-2 and dexamethasone, analysis of variance was used to investigate the effects of various factors. The model employed was:

$$X_{ijklm} = \mu + D_i + F_{j(i)} + T_k + I_e + DT_{ik} + DT_{ie} + TI_{ie} + DTI_{ike} + E_{ijklm} \quad ,$$

where D_i + effect of drug concentration $F_{j(i)}$ = nested effects of flasks, T_k = IUdR time effect, I_e = incubation time effect, and the other terms are the interaction effects. Note that the interaction effects with flasks are assumed zero. For the bone marrow study, the model assumed for the analysis was:

$$X_{ijklm} = \mu + D_i + F_{j(i)} + S_{k(i,j)} + T_e + DT_{ie} + E_{ijklm} \quad ,$$

where D_i = effect of drug concentration, $F_{j(i)}$ = nested effects of flasks, $S_{k(i,j)}$ = nested samples effect, T_e = IUdR time effect, and DT = dose and time interaction effect. The interaction effects with flasks and samples are assumed zero.

Selected results are being presented below: Figures 21 and 22 graphically presented the effect of TCE-2 (10^{-4}M = 10^{-2}M) on bone marrow cell number. The mean cell count in the 3 flasks containing no TCE-2 decreases from a 2×10^6 cells/ml at time 0 to 1.3×10^6 cells/ml over the 48-hour time period. This represents a 35% cell loss to the media. The TCE-2 effect on cell number is better represented in Figure 22, where the cell count is presented in the same block with the TCE-2 concentration. The bars show a dose-dependent decrease in cell number at $10^{-2.5}$M and 10^{-2}M at 15 minutes, 1 hour, and 3 hours. It should be noted that the vehicle group is the mean of 18 determinations, three flasks (A, B, C), three samples, and duplicate Coulter counter readings.

<u>Statistical Analysis</u>:

1. The effect caused by TCE-2 on bone marrow cell number is significantly decreased $p < 0.001$.
2. The interflask variation is significant at the $p < 0.001$ level. The estimated standard deviation for the flask is 140.
3. Concentration dependency is presently being analyzed.

Figure 21
Cell Counts

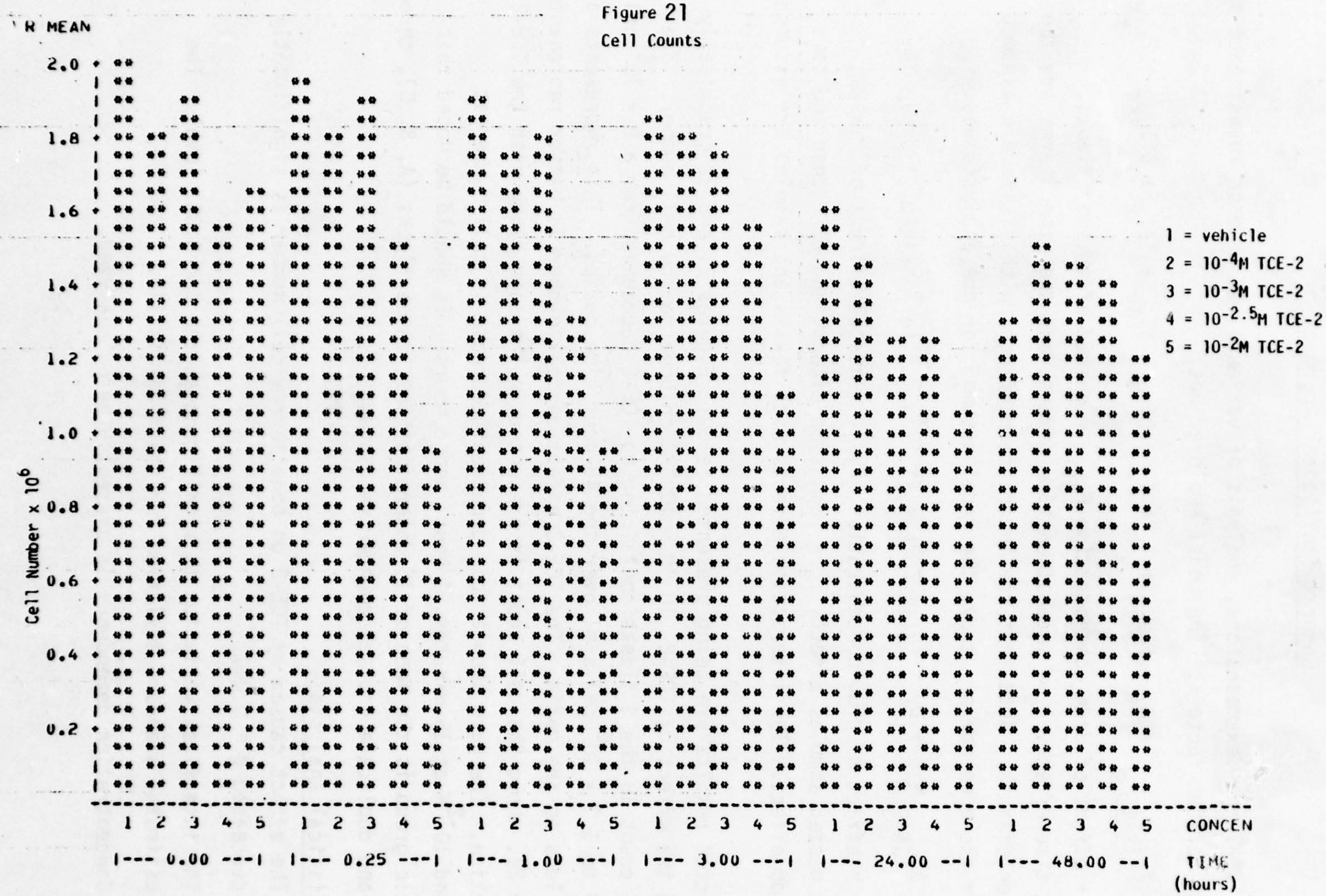
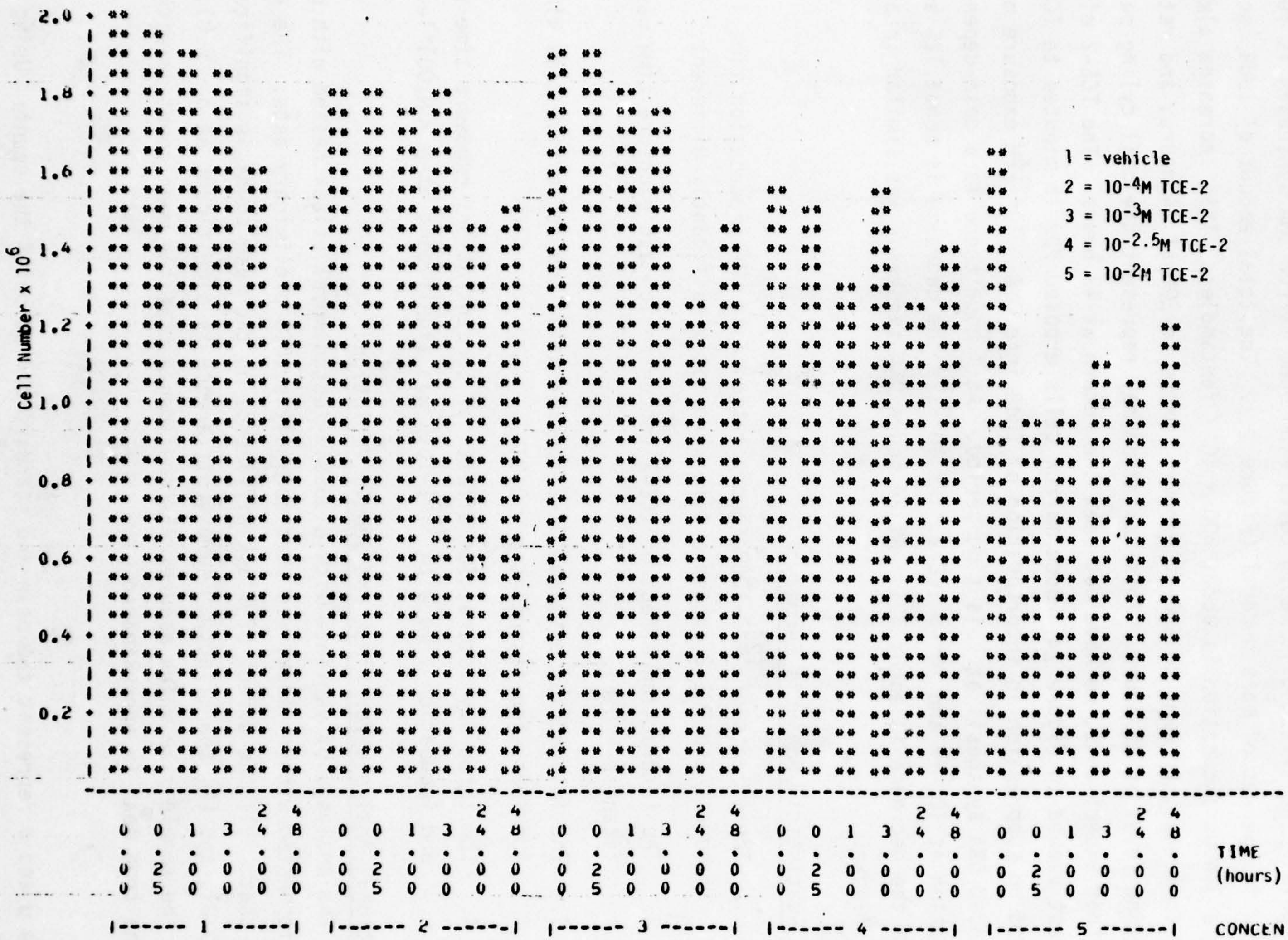


Figure 22
Cell Counts



Figures 23 and 24 graphically represent the effects of TCE-2 on bone marrow DNA synthesis. The effect of time in culture on bone marrow DNA synthesis is represented in the set of bars under 1 (Figure 23). The total amount of IUdR incorporated over a 3-hour incubation is about 600 x 10^{-3} fentamoles. This increases almost two-fold in 1 hour, returns to 630 at 3 hours, drops to 200 at 24 hours, and returns to baseline level at 48 hours. This variation may represent the cell cyling pattern. In other experiments, we have not seen the return at 48 hours. The TCE-2 effect is best viewed in Figure 24. Bone marrow cells exposed for 15 minutes to TCE-2 showed no suppression in incorporation of IUdR into DNA. $10^{-2.5}$M exposure markedly enhanced DNA synthesis at this time period. At 1 hour there is a dose-dependent reduction at $10^{-2.5}$ and 10^{-2}M TCE-2. At $10^{-2.5}$M the decrease is about 12% and at 10^{-2}M the decrease is about 72%. At 24 hours DNA synthesis was similar in all exposure flasks.

<u>Statistical Analysis Summary</u>:

1. The effects of ^{125}I-IUdR incorporation into DNA (incubation time 1, 2, 3 hours) and TCE-2 exposure time were not significantly different.
2. The interaction effect of TCE-2 concentration and exposure time was significant $p < 0.01$.
3. The interaction between TCE-2 concentration and incubation time with ^{125}I-IUdR was significant at $p < 0.01$.
4. The three-way interaction effect of concentration, exposure time to TCE-2, and incubation time with ^{125}I-IUdR was significant at $p < 0.001$ level.

<u>Additional Analysis for Flask and Sample Variation</u>:

The analysis of variance with a doubly nested model (flask nested with dose, sample nested within flask) was used to analyze this preliminary data. The results show that: (1) the effect of flask difference on the mean count is significant; $p < 0.01$, and (2) the effect of sample difference is significant at $p < 0.01$.

The sample size which assures 95% confidence that the mean count is within σ% of the true mean is approximated by

$$\frac{(1.96)^2\sigma^2}{(\sigma\mu/100)^2}$$

where μ and σ represent the mean and standard deviation of the count. Using the sample estimates, we approximate the required sample size as follows:

	5%	10%
Flask	7	2
Sample	7	2
Reading	4	1

Figure 23

DNA Synthesis in Bone Marrow Cells

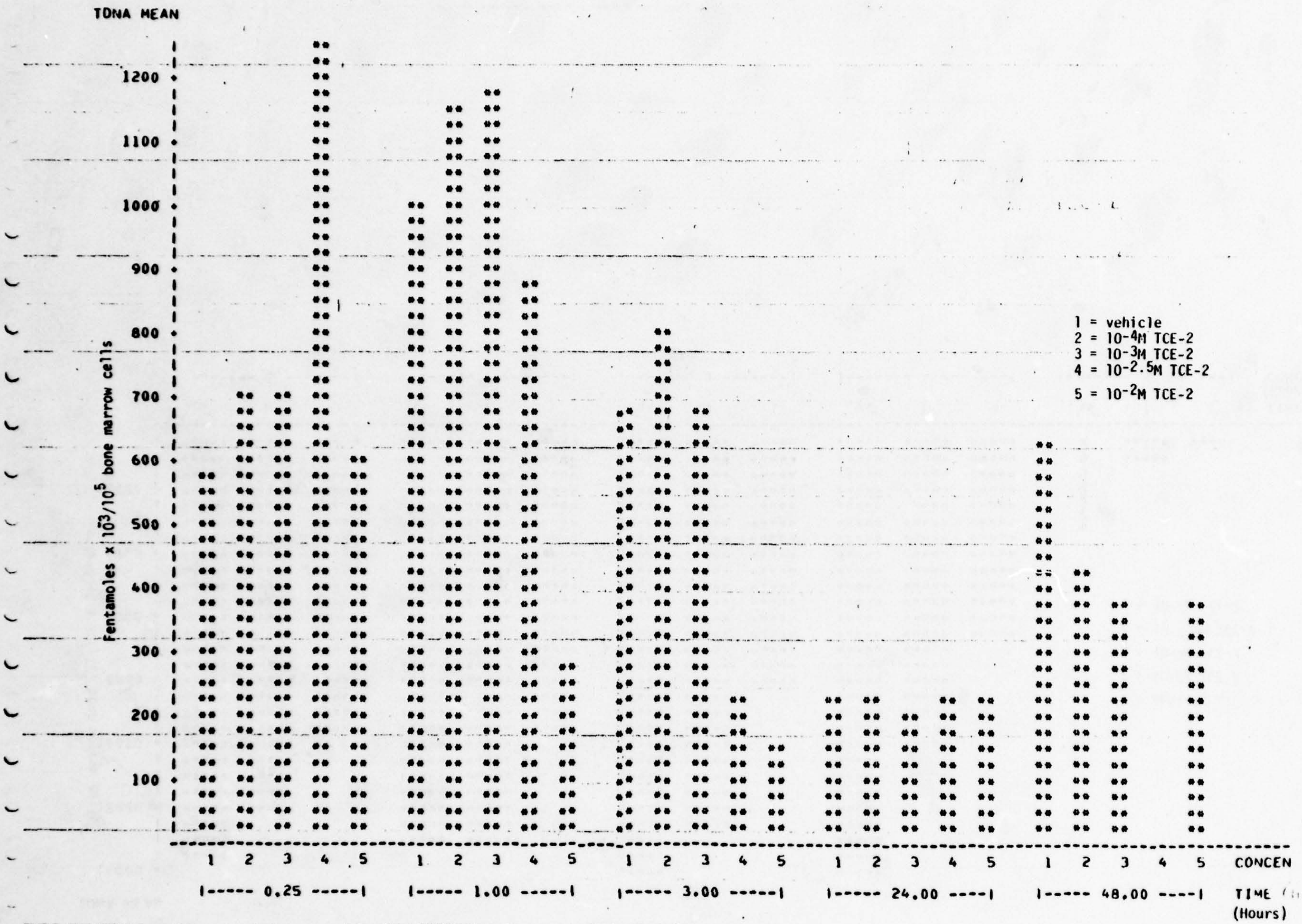
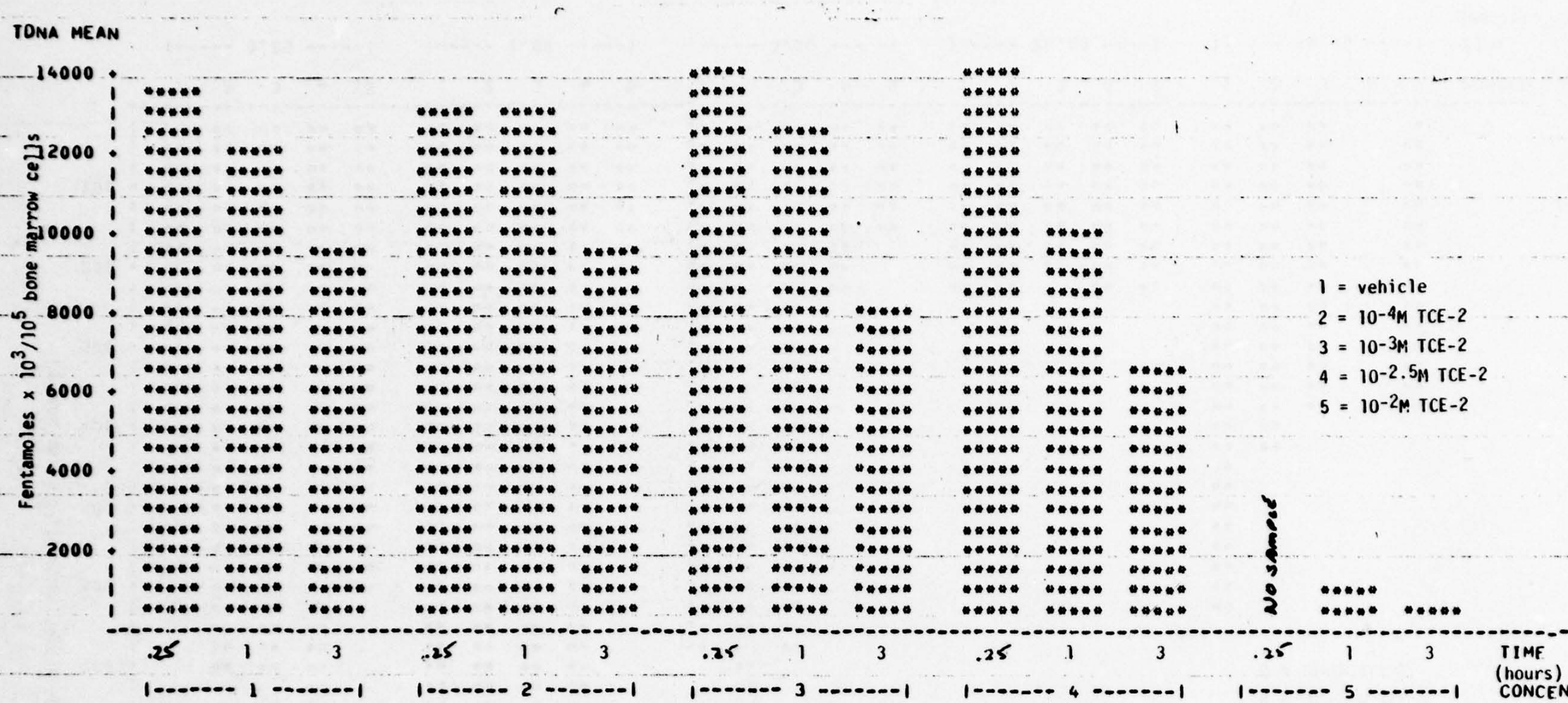


Figure 24
Bone Marrow DNA Synthesis



Calculated for the three flasks, the percent error was 7.8% for interflask variation, 6.6 for sample size, and 5.4 for Coulter counter replicates.

Figure 25 shows another experiment with the same design. Only DNA synthesis is presented. A dose- and time-dependent TCE-2 induced depression of DNA synthesis is seen.

Bone marrow stem cell growth from samples taken over the 48-hour time course was performed. Table 58 shows the data. Complete analysis is now being performed. There is a dose-dependent inhibition of 30% at 10^{-4}M, 48% at 10^{-3}M, 52% at $10^{-2.5}$M, and 52% at 10^{-2}M after 48 hours incubation with the chemical. It is interesting that the number of stem cells increases as a function of time in the untreated flasks. This may be a function of cell selectivity whereby non-colony forming cells are lost to adherence or to lysis. As indicated earlier, the cell number decreases over time and since the number of cells plated is maintained constant because of density dependency, it could very well be that the procedure selects for stem cells. The effect of TCE-2 in the experiment is not as dramatic as shown in other experiments where concentrations as low as 10^{-5}M produced greater than 50% inhibition (Table 50).

Returning to the question of cell adherence to the flasks, at the end of one flask exposure experiment, the flasks were rinsed three times with media and 2% trypsin added, incubated for 30 minutes and cell counts per flask determined. The results are shown below:

Flask	Total Cell number	
A vehicle	0.39×10^6	
B vehicle	0.46×10^6	0.39×10^6
C vehicle	0.33×10^6	
D 10^{-4}M trichloroethylene	0.66×10^6	
E 10^{-3}M trichloroethylene	0.84×10^6	
F $10^{-2.5}$M trichloroethylene	1.04×10^6	
G 10^{-2}M trichloroethylene	1.63×10^6	
H 10^{-2}M trichloroethylene	1.69×10^6	1.69×10^6
I 10^{-2}M trichloroethylene	1.76×10^6	

These results show a dose-dependent trichloroethylene effect on increasing the number of adhering cells. The total number of cells added to the flasks is 2.1×10^8. At the highest trichloroethylene concentration, only 0.8% of the cells adhered. We

Figure 25

Bone Marrow DNA Synthesis

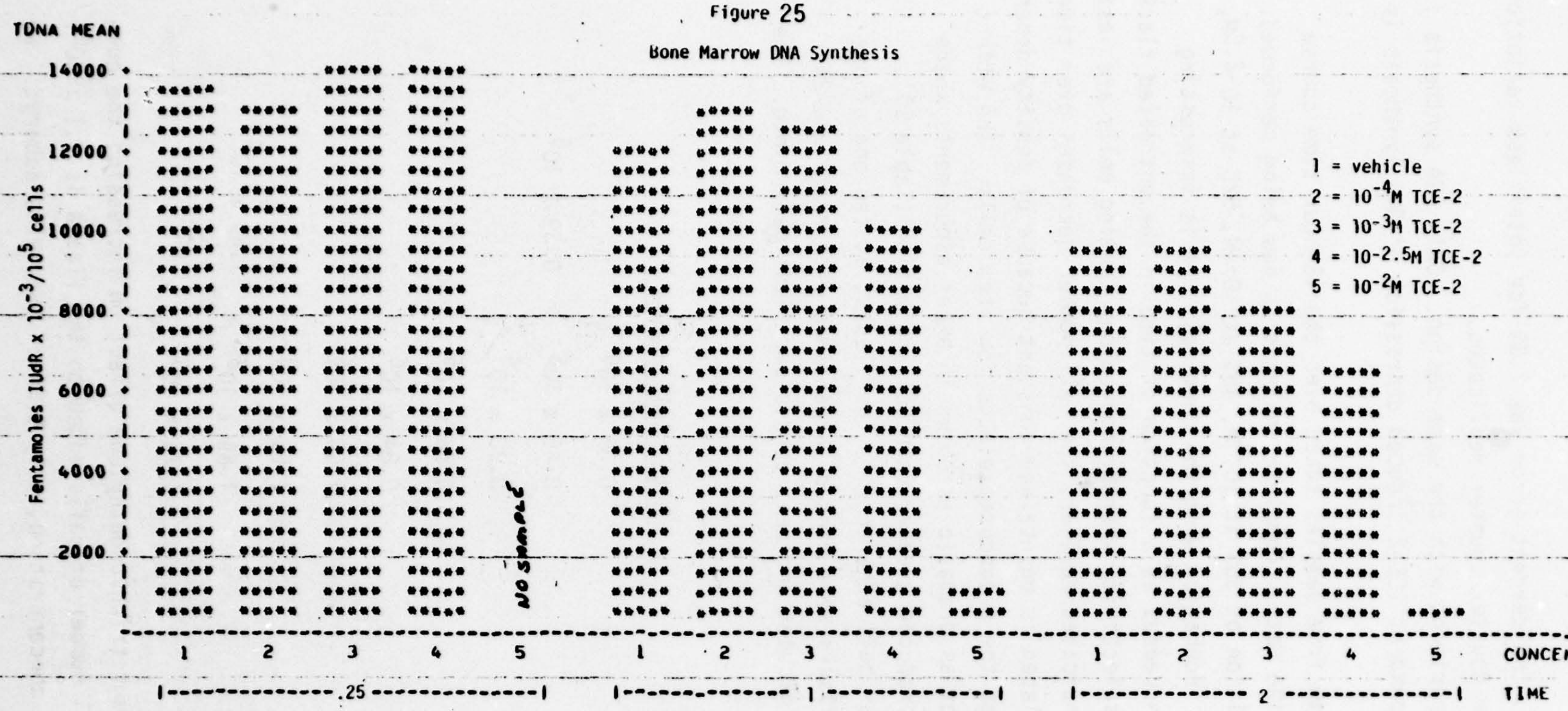


Table 58

Effect of Trichloroethylene on Bone
Marrow Stem Cell Formation

	Incubation (Hours)				
	0.25	1.0	3.0	24	48
Vehicle	98 ± 2	104 ± 2	99 ± 1	112 ± 2	146 ± 2
Vehicle	105 ± 1	101 ± 1	94 ± 1	109 ± 2	141 ± 2
Vehicle	111 ± 1	114 ± 1	155 ± 1	163 ± 3	201 ± 1
			116	128	162
10^{-4}M TCE-2	107 ± 2	111 ± 1	112 ± 1	131 ± 2	113 ± 4
10^{-3}M TCE-2	99 ± 1	105 ± 2	92 ± 1	109 ± 2	84 ± 1
$10^{-2.5}$M TCE-2	139 ± 2	176 ± 2	123 ± 1	91 ± 1	79 ± 1
10^{-2}M TCE-2	214 ± 3	173 ± 7	130 ± 5	101 ± 2	78 ± 2
10^{-2}M TCE-2	209 ± 2	159 ± 7	108 ± 5	103 ± 3	76 ± 2
10^{-2}M TCE-2	202 ± 3	164 ± 4	142 ± 7	102 ± 2	80 ± 2

Bone marrow cells were exposed to Trichloroethylene by the flask procedure at indicated concentrations for 48 hours. Triplicate samples taken at indicated times were plated and colony forming ability determined. Three flasks were used for vehicle and high concentration of TCE-2. Mean ± standard error derived from triplicate samples. Analysis is being performed.

are not sure that the trichloroethylene is not altering the plastic surface which enhances cell adherence. Although this is not many cells, it may represent a significant number of stem cells which could account for the shape of the time curve for stem cell growth.

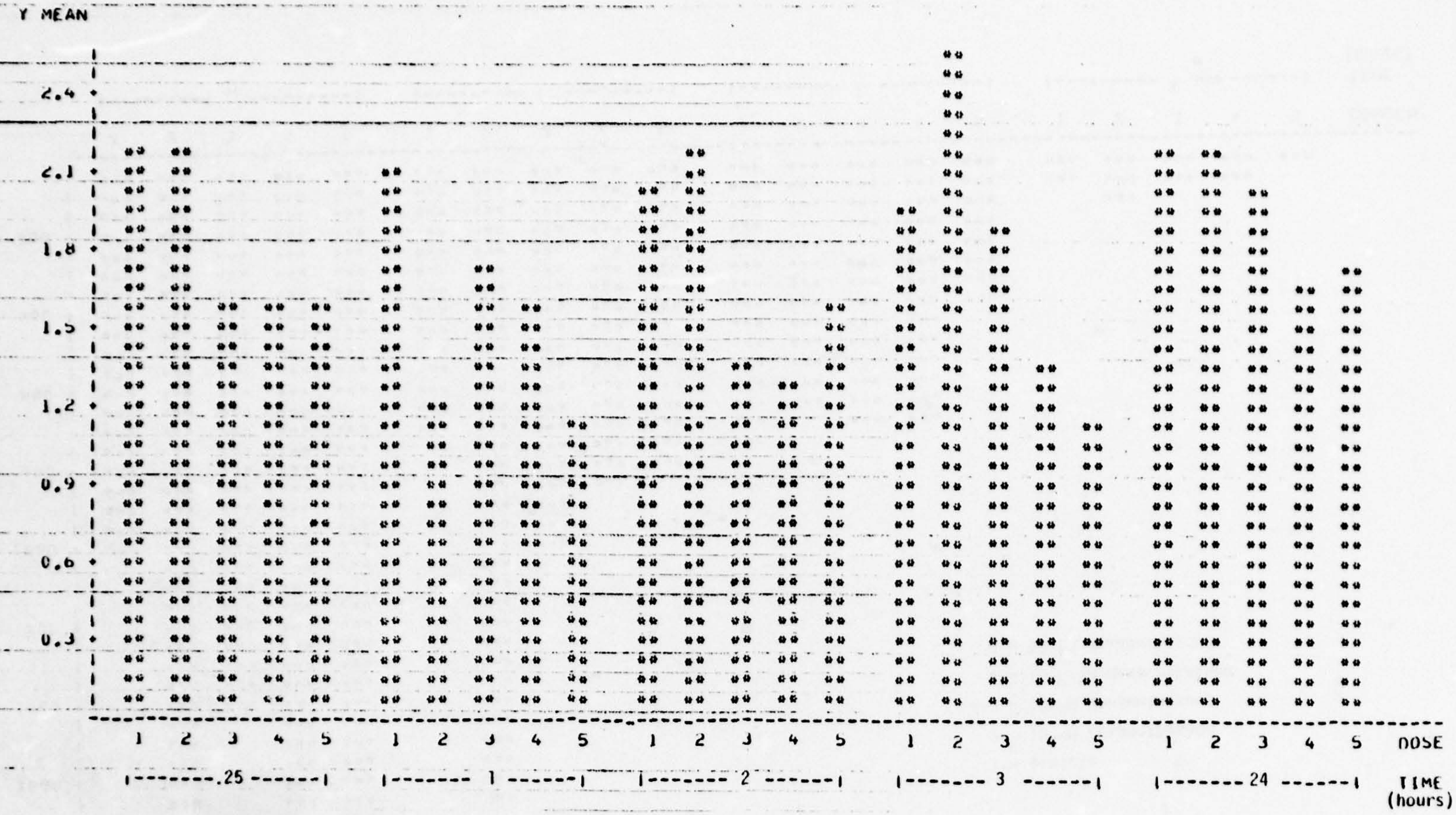
One last example of the in vitro exposure procedure. In this experiment, dexamethasone, a water soluble steroid, was used. Figure 26 shows the effect on bone marrow cell number as a function of concentration and time, while Figures 27 and 28 show the effect of DNA synthesis. Dexamethasone does not have the dramatic effect on bone marrow cells that TCE-2 possesses.

Statistical Analysis

1. The effect of dexamethasone concentration on mean cell count is not significant at $p < 0.05$.
2. The interflask variation is significant at $p < 0.001$.
3. The sampling variation (cell count) was marginally significant at $p \sim 0.05$.
4. The effect on cell number as a function of exposure time was not significant.
5. The interaction effect, i.e. dexamethasone concentration and exposure time as related to cell number, was significant. The cell number increased (?) as a function of time. $P < 0.005$.
6. Interflask variation of ^{125}I-IUdR incorporation was significant at $p < 0.005$.
7. Incubation time with ^{125}I-IUdR is significant at $p < 0.001$.
8. The exposure time to dexamethasone did not significantly alter ^{125}I-IUdR uptake.
9. The interaction effect of exposure time to dexamethasone and incubation time with ^{125}I-IUdR was significant at $p < 0.001$.
10. The three-way interaction effects, i.e., dexamethasone concentration, exposure time to dexamethasone, and incubation time with ^{125}I-IUdR was significant at $p < 0.005$.

This approach, i.e. performing several assays on one population of cells, is working out quite well. One experiment can be performed every 2-3 weeks. In the interim, continued refinements of the assays can be accomplished and data analysis completed.

Figure 26
Cell Counts



1 = vehicle

2 = 10^{-4}M dexamethasone

3 = 10^{-3}M dexamethasone

4 = $10^{-2.5}$M dexamethasone

5 = 10^{-2}M dexamethasone

Figure 27
Bone Marrow DNA Synthesis

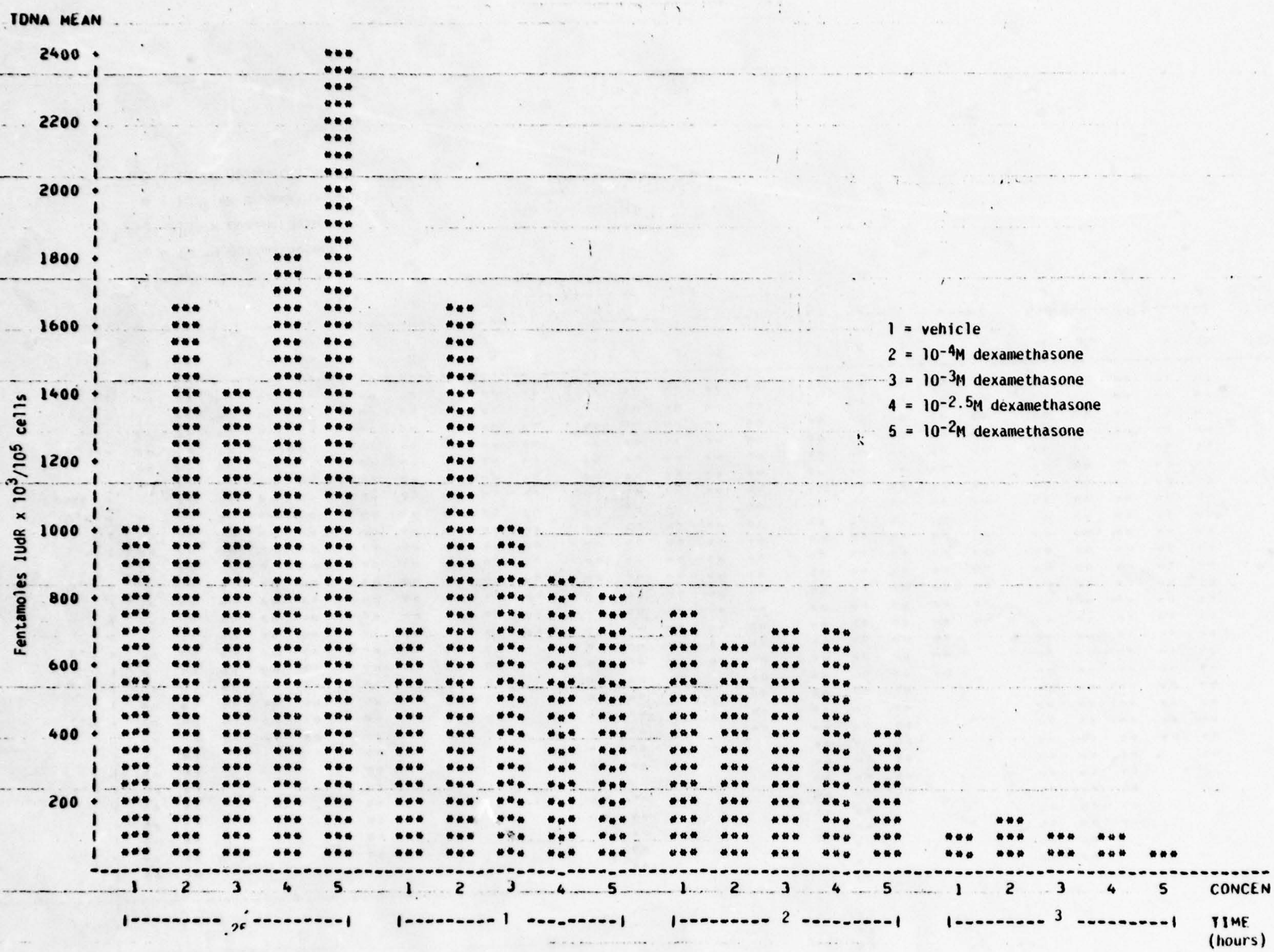
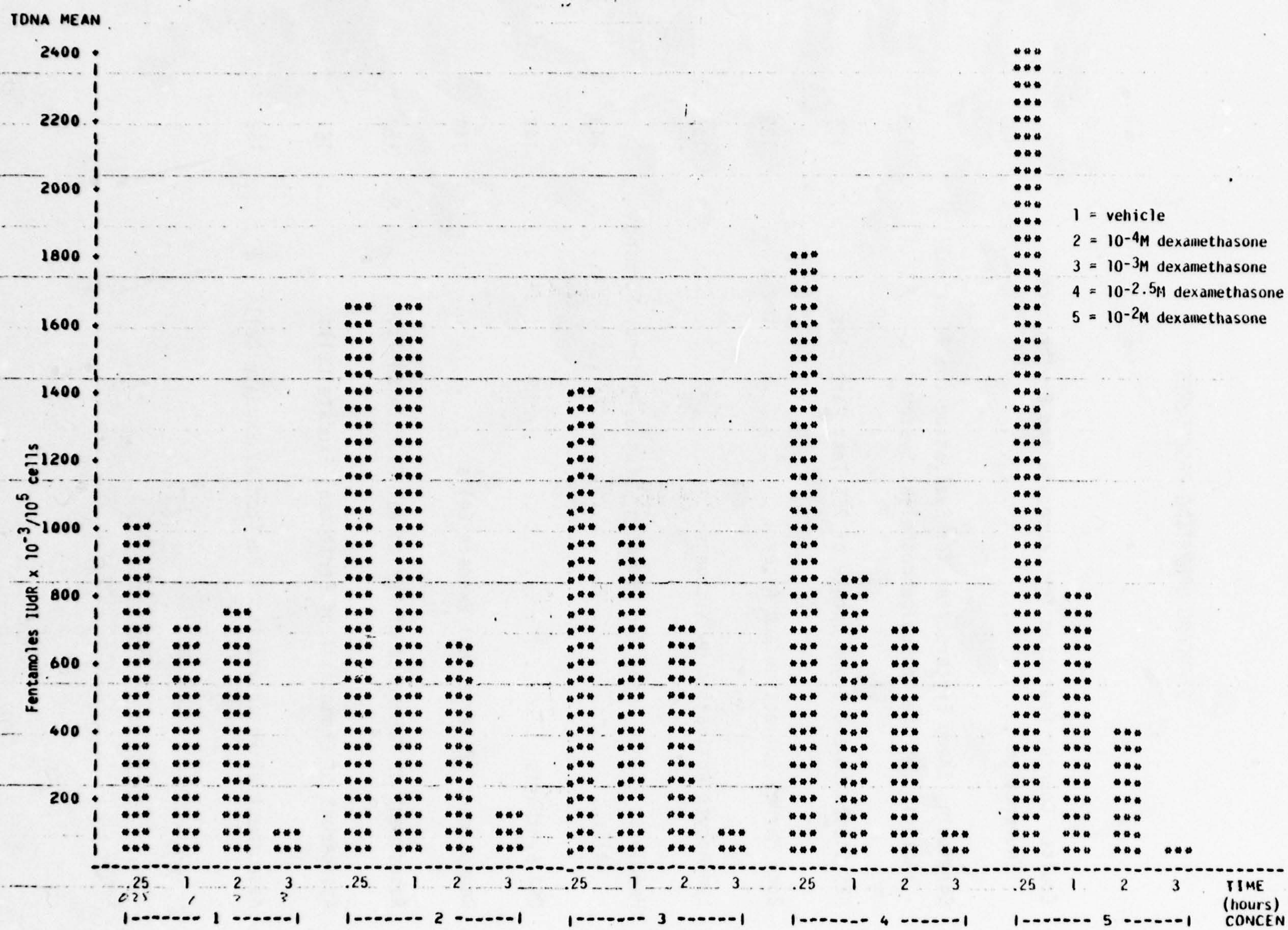


Figure 28
Bone Marrow DNA Synthesis



STANDARD OPERATING PROCEDURES

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APPENDIX

STANDARD OPERATING PROCEDURES

Title: Coulter Counter Operation for Leucocyte and Erythrocyte Enumeration

Prepared by: Barbara Kipps

Date: March, 1978

Purpose: To count numerous samples quickly and accurately using the Coulter count

Personnel:

1. One or two laboratory technicians trained in the procedure. If a large number of samples is to be counted, it is most efficient to have one person operate the machine and another record the data.
2. One technician who knows about the Coulter counter and would be available in case an equipment problem arises.

Materials and Equipment:

1. Coulter counter, model ZB1
2. Isoton
3. Dilution vials, prelabeled with group and animal number on top

Procedure:

1. Place 20 µl of blood in 10 ml of Isoton and agitate gently. Put 100 µl of this solution in 10 ml of Isoton in a second dilution vial. The first and second solutions are used for leucocyte and erythrocyte counts respectively.

2. Fill Isoton reservoir attached to the Coulter counter and be sure waste reservoir is empty.

3. Turn Coulter counter on by pulling knob on the lower left-hand corner. Pull down the white platform and place a vial of about 10 ml of Isoton on it. Carefully raise the platform, making certain the manometer is in the liquid. Set the amperage, lower threshold, and aperture at the values specified for the type of cell to be counted (e.g. Mouse RBC). These settings will vary from cell to cell and from machine to machine. Turn the upper right-hand stopcock to a vertical position. When a green line appears in the bottom of the sizing window, turn stopcock back to a horizontal position. The machine will then give a count (the background) that is acceptable if under 100 (for WBC) or 200 (for RBC). If these limits are exceeded, a fast flush must be made. Replace the vial of Isoton with one of detergent. Turn the top right and then the bottom left stopcocks to vertical positions. After approximately 30 seconds turn them back to horizontal positions in reverse order. Place a vial of fresh Isoton in the machine and repeat the fast flush. Now take a second background reading. If the reading is still above the limits, repeat the fast flush procedures with detergent and Isoton until a acceptable background is obtained. You are now ready to count samples.

4. For Acceptable Leucocyte Counts: Put 3 drops of red blood cell lysing ager in the vial of blood solution and gently mix, allowing at least 15 seconds for lysis to occur. Place vial on machine. Turn the upper stopcock to a vertical position until the green line appears and then turn it horizontally. While it is counting, prepare the next vial with lysing agent. Do not prepare vials with lysing agent too far ahead,

or leucocytes will also be lysed. The line will disappear when the count is finished. When the numbers displayed at the top left of the machine are 0 9 4 3 8, the actual leucocyte count is $9.438 \times 10^3/mm^3$. Remove the vial, empty into the dump beaker, and discard. Put the next prepared sample on the machine and repeat process.

5. For acceptable erythrocyte counts: Gently agitate the cell suspension (do not use lysing agent) and count on the machine. When the display reads 0 9 4 3 8, the actual erythrocyte count is $9.438 \times 10^6/mm^3$. Periodically, about every 20 samples, check background and if elevated perform a fast flush with detergent and then with Isoton until the background count is acceptable. Periodically (every 1-2 days or after 200-300 counts) a half-and-half solution of Isoton and Chlorox should be fast-flushed through. Leave the manometer in a vial of either detergent or Isoton. Turn off machine. Empty waste containers and fill Isoton reservoir.

6. Notes:
   (a) While counting, keep an eye on the debris window. If a piece of trash lands on the orifice, lower the platform with the vial and rub the orifice with your finger. Raise the platform and if the orifice is clear, take another count.

   (b) If a digit appears in the far left space of the display window, coincidence charts prepared by the Coulter Company must be used to determine the actual count. If 1 8 3 4 2 is displayed, look up 183 on the chart. To the right will be 192 and the count would be $19.2 \times 10^3/mm^3$ (leucocytes) or $19.2 \times 10^6/mm^3$ (erythrocytes).

   (c) If a large number of samples is being counted, perform a background count about every 20 samples.

   (d) For greatest efficiency and accuracy, perform cell counts on one type of cell before resetting instrument for another cell type.

Title: Chromating Sheep Erythrocytes for Evaluating the Functional Activity of the Reticuloendothelial System

Prepared by: Bernadine Kauffmann

Date: June 26, 1978

References: Greaves, M.F., et al., Lancet i, 68, (1969)

Principle: Cells are capable of incorporating chromium with which RES, organ uptake, and blood clearance can be measured.

Materials and Equipment:

1. Sterile laminar flow hood
2. Na chromate - 51
3. Sheep erythrocytes (sRBC) - 5×10^9 cells/ml
4. 17 x 100 test tube
5. Shaker bath
6. 50-ml conical tube
7. Alsevers solution
8. Capillary tube
9. Dulbecco's phosphate buffered saline (PBS)

Procedure:

A. General Guidelines:

1. Keep everything sterile by using laminar flow hood.
2. Use 1 mCi of ^{51}Cr for each 10 ml of sRBC.
3. If ^{51}Cr is from new bottle, count 100 μl after ^{51}Cr has been added to sRBC.
4. If sRBC are one week old, wash once in Alsever's and bring up to original volume. However, it is best to use fresh cells.

B. Incorporation:

1. Combine 10 ml of sRBC with 1 mCi ^{51}Cr in a 17 x 100 test tube.
2. Incubate tube in a shaker bath at 37°C for 30 minutes. Hand shake every 5 minutes.

C. Removal of Free Chromium:

1. Transfer sRBC with ^{51}Cr to a 50-ml conical tube after incubation.
2. Bring volume up to 40 ml in Alsever's Solution.
3. Centrifuge at 2200 rpm for 5 minutes at 5°C.

4. Remove plasma by aspiration and resuspend cells in Alsever's.

5. Wash cells 3 times in all. Before removing supernatant of final wash, count a 100-µl sample in gamma counter. If CPM are above 1500, wash two more times.

D. Adjustment of CPM:

1. Add 10 ml of cold cells from nonradioactive stock of 5×10^9 cells/ml. Bring volume up to 32 ml with Alsever's.

2. Take 100 µl and count; CPM should be 200,000. If too high, add about 2 ml more of cold cells and count again.

E. Hematocrit:

1. Use a capillary tube to take a sample. Seal one end with critoseal.

2. Centrifuge in microcentrifuge for 3 minutes. Read hematocrit: it should be close to 12%. If it is lower than 12%, centrifuge and take off about 2 ml of supernatant. Resuspend cells and take another sample. If the hematocrit is higher than 12%, add more Alsever's.

F. Final Preparation:

1. Mark line at top of Alsever's. Wash cells once in sterile PBS.

2. Resuspend in sterile PBS to line marked. Cells are now ready for use in RES.

G. Storage:

1. Cells can be stored overnight at 12% hematocrit in Alsever's. The next day, wash cells in sterile PBS.

Title: The Reticuloendothelial System (RES) Clearance and Organ Uptake of RES Test Particles

Prepared by: Beverly Barrett

Date: March 28, 1978

Purpose: To test the ability of the fixed macrophage system to phagocytize a radiolabelled particle.

Materials and Equipment:

1. Sheep red blood cells (sRBC) tagged with Cr^{51} to give 250-300,000 counts/.01 ml when sRBC are in PBS with a hematocrit of 12% (See procedure for chromating sRBC).
2. Mice
3. Cages
4. Heating pads
5. Bedding
6. Petri dish
7. 10X Bo-Lab pipette
8. 12 mm x 75 mm culture tube

Procedure:

1. Number, weigh, and place animals in individual cages on heating pads.

2. Clip tails. If blood counts, differentials, and/or hemoglobin are to be done, take the samples at this time.

3. At time zero give each animal an intravenous injection of the chromated sRBC (.1 ml/10gm).

4. Take blood samples from the tail of each mouse at predetermined time points. Using a 10X Bo-Lab pipette, collect the samples from a petri dish and dispense into a 12 mm x 75 mm culture tube containing 1 ml water, which is used to help rinse the pipette.

5. Decapitate the animals at sixty minutes and necropsy them.

6. Weigh the thymus, lungs, liver, spleen, and kidney and place in separate tubes to be counted.

Methods of Evaluation:

1. Count all samples in a Gamma Counter for 5 minutes at 3% error.

2. Use the Log Linear Regression program to calculate K or the Phagocytic Index.

3. Use the Organ Clearance program to determine the percent of body weight, the percent CPM absorbed, and the CPM/mg tissue.

4. Do an analysis of variance among all the groups if the F value is significant.

5. The Dunnett's T Test is used to determine where there is a difference between the control and experimental groups.

Sources of Error:

1. Wash the sRBC just prior to the experiment to rid it of free chromium, until the supernatant is below 1,000 CPM. If there is free chromium present, it will be reflected in a high uptake in the kidneys.

2. Keep the animals on the heating pads, in cages containing a small amount of bedding, at least 15 minutes to facilitate bleeding.

3. Keep the sRBC at room temperature throughout the experiment.

4. It is worthwhile to run a test clearance with the sRBC to see that you are getting a phagocytic index of about .04-.08. If not, you can decrease the hematocrit for a slower clearance or increase the hematocrit for a faster clearance. If a time point is missed, note it.

5. Take care with the pipette that the correct amount is drawn up and thoroughly rinsed.

6. Check the balance during weighings to see that the background is below 5 mg.

Example of Data:

Minutes	CPM
2	3375
4	2380
6	1504
8	943
10	474
15	149

K - .077

Title: Bone Marrow Collection and Assay

Prepared by: Patricia Hallett

Date: January 5, 1978

Purpose: To develop a sensitive immunotoxicological assay to be used in chemical testing situations.

Materials and Equipment:

1. Mice
2. Underpads
3. Sterile gauze
4. Gloves
5. Forceps
6. Surgical scissors
7. Plastic centrifuge tubes
8. Pipettes
9. 3-ml syringes
10. 25-gauge needles
11. Coulter Counter: Model ZBI
12. Magnetic stirring bar } optional
13. Stir plate } optional
14. 6-well Linbro plate
15. Hemocytometer and cover slip
16. 95% ethanol and alcohol burner
17. Trypan blue (0.4%)

18.	CMRL 1066 (10x) (Gibco)	20%
	Fetal calf serum	10%
	Horse serum	5%
	Sodium pyruvate	2.5%
	Non-essential amino acids	2.5%
	Essential amino acids	2.0%
	7.5% $NaHCO_3$	6.0%
	L-Serine (21 mg/ml)	0.05%
	L-Glutamine (200 mM)	1.0%
	L-Asparagine (10 mg/ml)	0.4%
	Minimal essential media vitamins (100x)	1.0%
	Vitamin B_{12}	0.25%
	Gentamicin	1.0%
	Colony Stimulating Factor	10%
	Enough distilled water to achieve desired volume	

19.	α-MEM (GIBCO)	70%
	Fetal calf serum	10%
	Horse serum	5%
	7.5 $NaHCO^3$	5%
	Colony stimulating factor	10%

Procedure:

A. Collection

1. Sacrifice mice by cervical dislocation and soak the lower half of their bodies in 95% ethanol.

2. Remove femurs and clean off as much surrounding tissue as possible.

3. Cut off both ends of the femur and, using a 25-gauge needle with CMRL or α-MEM from a 3-ml syringe, flush out marrow into a plastic centrifuge tube; break up any existing cell clumps by drawing them up and down in a Pasteur pipette.

4. Spin bone marrow suspension at 1000-1250 rpm for 8-10 minutes.

5. Pour off supernatant and wash cells twice in either enriched CMRL or α-MEM, depending on technical preference.

6. After final wash, resuspend cells in 1 ml of chosen media (CMRL or α-MEM) and count by either of two methods:
   a) trypan blue exclusion test
   b) Coulter Counter

7. Adjust cell counts to a concentration of 1×10^6 cells/ml.

B. Assay

1. Dilute cell suspension containing 1×10^6 cells/ml 1:10 in either CMRL or α-MEM.

2. Add either of the above mixtures to a suspension of methyl cellulose and water to obtain a final methyl cellulose concentration of 1.8% in the growth media.

3. Suspend cells evenly throughout media using a magnetic stirring bar on a stir plate or by pipetting the mixture up and down several times vigorously.

4. Using a 6-well Linbro plate, plate out 2 ml of the cell suspension per well.

5. Incubate plates at 37° C, 100% humidity, and 10% CO_2 for at least 7 days, and then count the cells via inverted microscope.

6. Calculate the number of colonies formed from the 10^5 cells that were plated out.

References:

1. Baum, M., Fisher, B., "Macrophage Production by the Bone Marrow of Tumor-Bearing Mice," Cancer Res. 32:2807-2812, (1972).

2. Fisher, B., Wolmark, N., "Correlation of Antitumor Chemoimmunotherapy with Bone Marrow Macrophage Precursor Cell Stimulation and Mø Cytotoxicity," Cancer Res. 36:2241-2247 (1976).

3. Lin, H., Stewart, C., "Peritoneal Exudate Cells," J. Cell Physiol. 83:369-378.

Title: Lymphocyte Activation by Mitogens

Prepared by: Virginia M. Sanders

Date: June 27, 1979

References: Greaves, M. and Janossy, G. Transplant Rev., Vol. II, 1972, pp. 87-130.

Janossy, G. and Greaves, M. Clin. Exp. Immunol., Vol. 9, 1971, pp. 483-498 and Vol. 10, 1972, pp. 525-536.

Principles: Gross morphological and biochemical characteristics of mitogen-induced lymphocyte responses *in vitro* are very similar to antigen-induced immune reactions *in vivo*. It has been suggested that mitogens "bypass" the requirement for antigenic recognition and induce cells to undergo that pattern of responses normally dependent upon immunological activation. It is therefore considered that the lymphocyte activation phenomenon *in vitro* offers a tool for monitoring the immunological competence of lymphcytes from animals exposed to various chemicals or of lymphocytes exposed to chemicals *in vitro*. Lymphocytes can be stimulated *in vitro* to increase their synthesis of DNA. Morphologically the cells enlarge, usually accompanied by mitosis. This stimulation can be brought about by many agents, such as plant lectins or bacterial exo- or endotoxins. In the mouse, phytohemagglutinin (PHA) and Concanavalin-A (Con-A) activate primarily thymus-dependent (T) lymphocytes, whereas endotoxins stimulate thymus-independent (B) lymphocytes.

Materials and Equipment:

1. Concanavalin-A (Type III) (obtained from Sigma Chemical)
2. Lipopolysaccharide (*E. coli* 0111:B4, obtained from Difco Laboratories)
3. RPMI 1640 medium
4. Penicillin-streptomycin
5. L-glutamine
6. Heat-inactivated calf serum
7. Fluorodeoxyuridine (FUdR)
8. ^{125}I-iododeoxyuridine (IUdR)
9. 96-well microtiter plates
10. Sterile mesh screen
11. Rocker platform
12. Titertek cell harvester
13. Gamma counter

Procedure:

1. Mitogens can be placed in 96-well microtiter plates and frozen at -70° C. Prepare mitogens (Con-A and LPS) in sterile RPMI 1640 medium containing penicillin-streptomycin (4 ml/100 ml medium) so that desired concentrations per well are contained in 0.05 ml. Concentrations should be 1, 5, and 10 µg/well for Con-A and 1, 10, and 30 µg/well for LPS. Set up microtiter plates as follows (6 wells per concentration):

   Row 1 - 0.05 ml media - no mitogen

   Row 2 - 0.05 ml low-concentration Con-A

   Row 3 - 0.05 ml middle-concentration Con-A

   Row 4 - 0.05 ml high-concentration Con-A

Row 5 - 0.05 ml low-concentration LPS

Row 6 - 0.05 ml middle-concentration LPS

Row 7 - 0.05 ml high-concentration LPS

2. Remove spleens sterilely from animals and place in RPMI 1640 medium containing penicillin-streptomycin.

3. Prepare single-cell suspensions by pushing spleens through a sterile mesh screen with RPMI 1640 medium containing penicillin-streptomycin, L-glutamine, and 15% heat-inactivated calf serum. Adjust cell concentrations to 5×10^6 cells/ml.

4. Thaw microtiter plates containing mitogens at room temperature and place 0.1 ml of the spleen cell suspension in each well.

5. Place plates on a rocker platform for 10 min in a 37°C incubator with 10% CO_2.

6. Remove plates from rocker and allow to incubate for 48 hours.

7. At the end of 48 hours, pulse plates with 0.05 ml of a solution of 4×10^{-6}M FUdR containing 0.2 μCi ^{125}I-iododeoxyuridine/0.05 ml.

8. Rock plates for 10 minutes and then incubate for 18 hours.

9. Using a Titertek cell harvestor, collect cells on filters and count them in a gamma counter for 2 min and 2% error.

Title: Hemolytic Plaque Assay for Detecting Single Antibody-Forming Cells

Prepared by: Leonard S. Lambert

Date: March 10, 1978

References: Cunningham, A.J. and Szenberg, A. Further Improvements in the Plaquing Technique for Detecting Single Antibody-Forming Cells. Immunology, 14:599-600, 1968.

Jerne, N.D. and Nordin, A.A. Plaque Formation in Agar by Single Antibody-Forming Cells. Science, 140:405, 1963.

Principle: Injecting mice intravenously or intraperitoneally with sheep red blood cells (sRBC) or any antigen results in formation of antibodies to that foreign substance. A plaque is formed as a result of the release of hemolytin by a single antibody-forming cell in response to the intruding foreign substance.

Materials and Equipment:

1. Mice maintained on Purina laboratory chow and tap water diet
2. Test chemical
3. Sheep red blood cells (sRBC) from a stock supply in the lab that is drawn weekly from sheep maintained by the university. The sRBC were washed twice with Alsevers, then suspended in Alsevers.
4. Guinea pig complement (Flow Labs, Rockville, Md.)
5. Rabbit anti-mouse IgG antisera
6. E. coli lipopolysaccharide 0127:B8 (Difco, Detroit, Mich.)
7. Phosphate buffered saline (PBS)
8. Sterile RPMI 1640 medium with 1% penicillin and streptomycin and 2% glutamine
9. 5- or 10-ml test tubes
10. Rubber plungers from 10-cc syringes
11. Pasteur pipettes
12. Brass wire mesh screens
13. 60 x 15 petri dish (Falcon Plastics, Oxnard, Ca.)
14. Microscope slides
15. Double-sided tape (Scotch Brand No. 410)
16. Paraffin
17. Microtiter II test plate (Falcon Plastics, Oxnard, Ca.)
18. Dark-field colony counter (American Optical, Buffalo, N.Y.)
19. Coulter Counter

Procedures:

A. IgM Response of Hemolytic Plaque-Forming Cells to Sheep Red Blood Cells:

1. Immunize the mice with 4×10^8 sRBC i.p. on day 0.

2. Administer chemicals i.p. on day 2 and measure antibody-producing cells daily from day 3 to day 6.

3. On days 3 through 6 after immunization, weigh the mice and sacrifice them by cervical dislocation.

4. Remove the spleens asceptically, weigh them, and place them in 3 ml of RPMI 1640 medium in a test tube on ice. Prepare spleen cell suspensions by gently pressing the spleens with a rubber plunger from a 10-cc syringe through a wire mesh screen into a petri dish. Rinse the screen with a Pasteur pipette by aspirating the cell suspension into the pipette and then passing it back through the screen. Then place the cell suspension in the test tube.

5. Wash the cells twice in a centrifuge at 1000 rpm and 4°C for 10 minutes; resuspend them in 3 ml of fresh medium with the aid of a Pasteur pipette; count them on the Coulter counter; and adjust them to a constant cell concentration (2×10^6/ml) in RPMI 1640 medium.

6. Remove volumes of 25 µl, 50 µl, and 100 µl of cells from each adjusted spleen cell suspension and place into three separate wells of a Microtiter II test plate. To the well containing 25 µl of cells, add 105 µl of medium; to the 50-µl well, add 80 µl of medium; and to the 100-µl well, add 30 µl of medium. To each well, add 10 µl of 6×10^9/ml sRBC.

7. To enumerate IgG plaque-forming cells, add rabbit anti-mouse IgG antisera to the cell suspension at a dilution of 1:150, a concentration that inhibits IgM antibody-forming cells from forming plaques.

8. After the addition of the antisera, add 10 µl of guinea pig complement, making the total volume of each well 150 µl. Immediately after adding the guinea pig complement, mix the contents of the well with a long Pasteur pipette.

9. Immediately apply the cell mixture to a two-sided plaquing slide, which is formed by joining two glass microscope slides with double-sided tape. Seal the slides by dipping all edges into paraffin and incubate for 45 minutes at 37°C. Count plaques immediately after removal from the incubator under a dark-field colony counter.

B. Response of Hemolytic Plaque-Forming Cells to *E. coli* LPS:

1. Immunize the mice intravenously with 10 mg of 0127:B8 LPS on day 0.

2. Administer chemicals i.p. on days indicated by the protocol and enumerate plaque-forming cells on days 3 through 6.

3. On days 3 through 6, sacrifice the mice and prepare spleen cell suspensions as previously described.

4. Prepare LPS-coated sRBC in the following manner: boil a solution of 100 mg/ml LPS in phosphate buffered saline (PBS) in a 50-ml beaker covered with a petri plate (to reduce evaporation) for 1 hour. If the volume of the solution is less than enough to run the assay after boiling 1 hour, add PBS until the original volume is obtained. Let

the solution reach its boiling point again, and then let cool to 37°C. Incubate 2 volumes of 10% sRBC {obtained by adding 5 ml of 5.75×10^9/ml with 1 volume of the LPS solution (add 8.5 ml LPS to 17 ml of 1.7×10^9/ml sRBC)} for 1 hour in a 37° water bath. After incubating, wash the LPS-coated sRBC 3 times with Alsevers and adjust the concentration to 6×10^9/ml for use in plaquing as previously described.

Methods of Evaluation:

The statistical confidence for this experiment is determined by calculating plaque-forming cells for 10^6 spleen cells and calculating plaque-forming cells per spleen. If more than one experimental group is used, an analysis of variance may be used to compare the control group with the experimental groups.

Sources of Error:

Several critical errors can be made during the above experiment that will have a direct effect of the accuracy of data obtained. Not keeping the cells on ice causes lysis, thereby reducing the number of cells available to form plaques. Counting plaque-forming cells too soon does not allow time for a maximum number of plaques to form, whereas counting plaque-forming cells too late does not allow for the plaques that had formed and dissolved prior to counting.

Example of Data :

Gp	Body wt gms.	Spleen wt gms.	% Body wt.	10^6 cells/ml 3 ml.	10^7 cells/spleen	PFC/Slide 25	PFC/Slide 50	PFC/Slide 100	PFC/ 10^6	10^4 PFC/ spleen
1	21.3	.132	.62	8.4	2.52	10	24	53	235.0	.592
2	21.4	.046	.21	16.1	4.83	47	83	187	901.7	4.355
3	23.8	.040	.17	15.0	4.50	18	47	103	448.3	2.018
4	24.1	.068	.28	19.2	5.76	12	31	54	273.3	1.574
5	23.1	.082	.35	16.0	4.80	16	35	68	336.7	1.616
6	25.4	.069	.27	14.3	4.29	8	21	33	178.3	.765
$\bar{X}$	23.2	.073	.31	14.8	4.45	18.5	40.2	83.0	395.6	1.820
SD	1.60	.033	.18	3.57	1.07	14.4	22.9	56.0	264.7	1.356
SE	.655	.013	.08	1.46	.437	5.9	9.3	22.9	108.1	.553
% of Control	88.2	58.4	67.4	54.4	55.0	107.6	92.4	109.0	102.4	54.1

Title: DNA Synthesis

Prepared by: Bernadine Kauffman

Date: March, 1979

Principle: ^{125}I-IUdR incorporates into DNA; therefore, DNA synthesis can be measured.

Materials and Equipment:

1. Bone marrow or spleen cells
2. ^{125}I-iododeoxyuridine (IUdR) from New England Nuclear
3. Complete Spinners medium
4. 10^{-4}M cytosine-arabinose (Ara-C)
5. 2.0 x 10^{-5}M fluorodeoxyuridine (FUdR)
6. Cooke Microtiter "V" plates
7. Skatron multiple cell culture harvester and filter paper from Flow Laboratories
8. 0.85% saline
9. 12 x 75 mm tubes

Procedure:

1. Centrifuge cell samples from flasks at 1500 RPM for 10 min. Resuspend in Complete Spinners medium to original volume. Count cells.

2. Add 200 μl of cells to wells of microtiter plate.

3. Add 20 μl of IUdR at a concentration of 0.1 μCi/20μl in 2.0 x 10^{-5}M FUdR per well.

   a. FUdR stock is 5 x 10^{-3}M. Dilute 1:300 in Complete Spinners to obtain 2 x 10^{-5}M.

   b. If specific activity of IUdR is 500 μCi/ml or 10 μCi/20μ, dilute IUdR 1:100 in 2 x 10^{-5}M FUdR.

4. Add 20 μl Ara-C per well as a negative control for DNA synthesis.

5. Incubate cells with IUdR and FUdR for 30 min, 1 hr, 2 hrs, 3 hrs at 37°C and 5% CO_2.

6. Harvest cells on cell harvester, flushing wells with saline for complete removal of cells.

7. Place filters in tubes and count on gamma counter.

Method of Evaluation:

1. Find mean, standard deviation, and standard error of CPM. Convert mean CPM to CPM/10^5 cells. Then plot on linear graph paper CPM/10^5 cells versus the time of incubation with IUdR. This will determine the rate of incorporation and amount incorporated.

Sources of Error:

1. Pipetting wrong amount of radiolabel.

2. Diluting wrong amount of radiolabel.

3. Not rinsing wells enough times during harvesting.

4. Not pushing filters down in tubes (counter will not read filters efficiently).

Title: Recruitment of Peritoneal Exudate Cells

Prepared by: Fay Kessler

Date Prepared: August, 1979

Purpose: To recruit peritoneal exudate cells (PEC) for use in laboratory assays.

Personnel: One laboratory technician adept at sterile technique and handling animals.

Materials and Equipment:

1. ICR mice
2. Thioglycollate (TG)
3. Syringes and needles (preferably 20 gauge)
4. Dissecting instruments (forceps and scissors)
5. Dissecting board
6. 75% ethanol
7. Dulbecco's Minimal Essential Medium (DMEM)
8. 50-ml centrifuge tubes
9. Beckman TJ-6R centrifuge

Procedure:

1. Inject ICR mice (20-25 grmas) with 1 ml of 10% Brewer's thioglycollate (TG).

2. Sacrifice the mice by crushing the cranium 5 days after injection with TG. Do not sacrifice the animals by any other technique, as this technique minimizes the amount of blood leakage to the peritoneum.

3. Exposed to peritoneal cavity by pinning the animal, abdomen up, on a dissecting board. Paint the skin with 75% ethanol and then make a small midline incision. Grasp the skin with forceps and pull back to expose the peritoneal membranes with membranes intact.

4. Using a sterile syringe and sterile DMEM, inject 10 ml of medium into the peritoneal cavity. Remove the wash by gently aspirating the fluid from the peritoneal cavity. Use extreme caution to avoid nicking the bowel.

5. Put the peritoneal exudate cells in a sterile 50-ml centrifuge and wash once at 300xg for 10 minutes at 4°C in a Beckman TJ-6R centrifuge.

6. Discard supernatant and resuspend the pellet in fresh DMEM. Cells are now ready to be counted and used for the desired experiment.

Title: Assessment of Adherence of Peritoneal Exudate Cells

Prepared by: Richard A. Carchman, Ph.D.

Date: August, 1979

Materials and Equipment:

1. Peritoneal exudate cells (see Standard Operating Procedure for recruitment of peritoneal exudate cells).
2. 24-well plastic Costar dishes
3. CO_2 incubator
4. ZBI Coulter counter

Procedure:

1. Inoculate 24-well plastic Costar dishes with 2×10^5 peritoneal exudate cells per well and allow to incubate 18-24 hours in a 37°C humidified CO_2 incubator.

2. Wash the cells extensively, scrape them from the plate, then count the adherent cells in a Coulter counter.

Title: Assessment of Chemotaxis of Peritoneal Exudate Cells

Prepared by: Richard A. Carchman, Ph.D.

Date: August, 1979

Materials and Equipment:

1. Peritoneal exudate cells (see Standard Operating Procedure for recruitment of peritoneal exudate cells)
2. CO_2 incubator
3. Blind well Boyden chambers (Dept. of Surgery, Duke University, S.C.)
4. 13 mm Sartorius membrane filters, 5 μ pore size, 150 μ thick (Beckman Science Essentials, Somerset, N.J.)
5. Blunt-tipped forceps
6. Methanol
7. Formalin
8. Glass slides (Permount)
9. Metal staining clips
10. Hematoxylin stain
11. 0.1% NH_4OH
12. 95% ethanol
13. 100% ethanol
14. 100% 2-propanol
15. Xylenes
16. Nikon microscope interfaced with an Artec model 980 Image Analyzer

Procedure:

1. Perform assays in blind well Boyden chambers using 13-mm Sartorius membrane filters. Add chemoattractant solutions (or vehicle) to the well and center the membrane filter over it.

2. Place varying numbers of cells ($1\text{-}2 \times 10^6$ cells/ml) in the upper portion of the chamber (300 μl). Incubate the chambers at 37° C in a humidified incubator for 4 hours.

3. Remove cells from the top of the chamber, unscrew the cap, and remove the filter with blunt-tipped forceps.

4. Dip filters once in methanol, then place in formalin upside-down until stained.

5. Place the filters on glass slides (6/slide) using metal staining clips. Stain them with hematoxylin for 10 minutes, then rinse them with water until the rinse is clear.

6. Place the filters in a 0.1% solution of NH_4OH for one minute. Dip the filters in distilled water and drain them on a pad.

7. Following this, place the slides sequentially in 95% ethanol (5 minutes), 100% ethanol (3 minutes), and 100% 2-propanol (5 minutes). After each step, drain the filters.

8. Place the filters in xylenes until the filter is cleared (at least 5 minutes).

9. Mount the filters on glass slides, dry, and count 20 microgrid fields on a Nikon microscope interfaced with an Artec model 980 Image Analyzer.

Title: Assessment of Phagocytosis of Peritoneal Exudate Cells

Preapred by: Richard A. Carchman, Ph.D.

Date: August, 1979

Materials and Equipment:

1. Sheep red blood cells (sRBC)
2. Alsever's solution
3. Centrifuge
4. ^{51}Cromium ($Na_2{}^{51}CrO_4$, NEN)
5. Incubator
6. Antibody against sRBC (obtained from sRBC-immunized DBA/2 mice 10-12 days after sRBC administration)
7. Peritoneal exudate cells (see Standard Operating Procedure for recruitment of peritoneal exudate cells)
8. 24-well plastic Costar dishes
9. Dulbecco's Minimal Essential Medium (DMEM)
10. Eagle's Minimal Essential Medium (EMEM)
11. 5-ml test tubes
12. Pasteur pipettes
13. 1 N NaOH
14. Beckman 300 gamma counter
15. Phosphate buffered saline OR 3% lyzerglobin

Procedure:

A. Preparation of ^{51}Cr-Labelled Opsonized Sheep Red Blood Cells

1. Wash the sRBC with Alsever's solution until the supernatant is straw colored, indicating that most of the lysed sRBC have been discarded. Centrifuge the cells following washings at 500xg for 10 min. The initial volume of the sRBC is 15 ml of a 50% suspension.

2. Combine one ml (1 mCi) ^{51}Cr with 5 ml of sRBC (50% suspension in Alsever's solution), and incubate at 37°C for 30 minutes. During the incubation, the sRBC should be agitated gently every 5 min.

3. Wash the labelled sRBC. After the third wash, the counts/ml of supernatant are approximately 350 cpm, and after the seventh wash the counts usually decrease to around 250 cpm in 1 ml. Ten λ of the 50% sRBC suspension give counts in excess of 200,000 cpm.

4. Opsonize the ^{51}Cr-sRBC. First make a 1:10 dilution of the antibody against sRBC with Alsever's solution. Add this serum to the ^{51}Cr-sRBC to yield approximately 10 ml.

5. Incubate this suspension at 37°C for 30 minutes then wash twice with Alsever's solution. Return the cells to a 50% suspension in Alsever's solution for use and storage. Use the solutions within 24-48 hours.

B. Assessment of Phagocytosis

1. Add 2 x 10^5 peritoneal exudate cells in 1 ml DMEM to each well of a 24-well plastic Costar dish.

Annual Report

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Errata to Army Annual Report

TITLE: Development of Short-Term Immunotoxicological Assays for the Prediction of Chronic Toxicological Response Induced by Environmental Chemicals

AUTHOR: Albert E. Munson, Ph.D.

DATE: September, 1979

CONTRACT NUMBER: DAMD17-78-C-8083

The following corrections have been made and are to be attached to copies of the report.

a) The following words are to be added to the DD Form 1473 for Block 19: acute, cellular, cytotoxicity, emulphor, humoral, immunity, subchronic, toxicity.

b) Second paragraph of INTRODUCTION: change Mudel to MUUL.

c) Page 7 - additional references:

Christensen, H.E., Fairchild, E.J.(Eds.) Registry of Toxic Effects of Chemical Substances, 1976 Ed. Rockville, MD, U.S. DHEW, National Institute for Occupational Safety and Health.

U.S. Department of Health, Education, and Welfare. Carcinogenesis Bioassay of Trichloroethylene, CAS No. 79-01-6, National Cancer Institute Carcinogenesis Technical Report Series No. 2, February 1976, p.1.

d) Page 32, paragraph 3 - sentence should read: "This does not represent ..., ... control range for mice not exposed to TCE-2."

e) Page 46, paragraph 2, first sentence - delete "along with their water bottles."

f) Page 81, Table 37 - the unit for body weights is grams

g) Page 82, Table 38 - organ weights are presented as percent of body weight, and 1.3 μg/ml should be changed to 0.3 μg/ml.

h) Page 115 - The freeze-thaw experiment was performed once and each mitogen concentration was done using 6 microtiter wells.